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Investigating the Contribution of the Inflammasome to Persistent Pain States

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Investigating the Contribution of the Inflammasome to Persistent Pain States

Amanda Ellis

Thesis presented for the degree of:

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at King's College London

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Abstract

Neuropathic pain, a debilitating condition, occurs as a consequence of nerve injury with symptoms such as spontaneous pain, hyperalgesia, and allodynia commonly reported. In animal models of peripheral nerve injury, there is accumulation and proliferation of microglial cells in the injured spinal cord and macrophages within the injured nerve and dorsal root ganglion (DRG), contributing to the development of pain-like behaviours. The pro-inflammatory cytokine interleukin-1 β (IL-1 β) plays a key role in acute and chronic inflammation. It causes potent mechanical and thermal hyperalgesia when injected into peripheral tissues, and increased expression of IL-1 β in the spinal cord, DRG, and injured nerve is seen in several animal models of inflammatory and neuropathic pain. A key step in the release of active IL-1 β is the cleavage of pro-IL-1 β by active caspase-1, generating mature IL-1 β . Activation of this enzyme requires assembly of the inflammasome, a multi-protein complex. The active complex contains a central scaffold protein (eg nod-like receptor-1 (NLRP1), NLRP3), the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1. It is known to assemble in response to a variety of exogenous and endogenous danger signals (eg adenosine-5'-triphosphate (ATP), monosodium urate (MSU) crystals).

Data presented in this thesis demonstrates that, following L5 Spinal Nerve Transection (SNT), an animal model of neuropathic pain, inflammasome components NLRP3, ASC, and caspase-1 are upregulated in the injured DRG and the ipsilateral lumbar dorsal horn. Immunohistochemical analysis reveals that ASC and caspase-1 are both highly co-localised with macrophage and microglial marker ionised calcium-binding adaptor molecule-1 (IBA1), but not with astrocytic marker glial fibrillary acid protein (GFAP) or neuronal marker NeuN. While daily intrathecal administration of Ac-YVAD-CMK, a cell-permeant caspase-1 inhibitor, significantly attenuated established behavioural hypersensitivity following L5 SNT, ASC $^{-/-}$ and NLRP3 $^{-/-}$ mice developed behavioural hypersensitivity normally in the Partial Sciatic Nerve Ligation (PSNL) model of neuropathic pain, indicating an inflammasome-independent role of caspase-1 in neuropathic pain. In a model of central inflammatory pain,

pharmacological inhibition of caspase-1 prevented the development of hindpaw mechanical hypersensitivity following intrathecal administration of lipopolysaccharide (LPS). ASC^{-/-} mice had a significantly reduced mechanical hindpaw hypersensitivity following intrathecal administration of LPS. However NLRP3^{-/-} mice developed hindpaw hypersensitivity normally following intrathecal administration of LPS, indicating a NLRP3-independent role for ASC and caspase-1 activation in this animal model of central inflammatory pain. Finally, although ASC appears to contribute to the development of hypersensitivity in a model of central inflammatory pain, ASC^{-/-} mice developed behavioural hypersensitivity normally following intraplantar administration of complete Freund's adjuvant (CFA) in an animal model of persistent peripheral inflammatory pain.

Thus data presented in this thesis reveal a complex role for the inflammasome in animal models of pain, with differential contribution of inflammasome components to behavioural hypersensitivity in animal models of neuropathic and central and peripheral inflammatory pain.

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List of Abbreviations

5-HT	5-hydroxytryptamine
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APDC	(2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATF-3	Activating transcription factor-3
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BIR	Baculovirus inhibitor repeat
CAPS	Cryopyrin-associated periodic syndromes
CARD	Caspase activation and recruitment domain
CCI	Chronic constriction injury model of neuropathic pain
CCL3	Chemokine (C-C motif) ligand 3
CD14	Cluster determinant factor 14
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX	Cyclooxygenase
CR3	Complement receptor 3
CREB	cAMP responsive element binding protein
DAMP	Danger-associated molecular pattern
DRG	Dorsal root ganglion
ERK	Extracellular signal-related kinase
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GDNF	Glial-cell derived neurotrophic factor
HCN2	Hyperpolarisation-activated cyclic nucleotide-modulated 2
IB4	Isolectin B4
IBA1	Ionised calcium-binding adaptor molecule-1
ICE	Interleukin-1 converting enzyme (caspase-1)
IFN	Interferons

IL	Interleukin
IL-18BP	Interleukin-18-binding protein
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
INCA	Inhibitory CARD
iNOS	Inducible nitric oxide synthase
IRAK	IL-1R-associated kinase
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCP-1 / CCL2	Monocyte chemotactic protein-1 / Chemokine (C-C motif) ligand 2
MHC	Major histocompatibility complex
MMP	Matrix metalloprotease
MSU	Monosodium urate
MyD88	Myeloid differentiation primary response gene 88
NAC	N-acetyl-cysteine
NALP1/3	NACHT, LRR, and PYD-containing proteins 1/3 (now known as NLRP1/NLRP3)
NEMO	NFκB essential modifier (also IKK-γ)
NGF	Nerve growth factor
NK-1	Neurokinin-1 receptor
NLRC4	Nod-like receptor family CARD domain-containing protein 4
NLRP1	Nod-like receptor protein 1
NLRP3	Nod-like receptor protein 3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOD	Nucleotide oligomerisation domain
NPY	Neuropeptide-Y
NRG	Neuregulin
NT-3	Neurotrophin-3

P2X	Purinergic ionotropic receptors
PAG	Periaqueductal grey matter
PAMP	Pathogen-associated molecular pattern
PG	Prostaglandin
PI3K	Phosphoinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PRR	Pattern recognition receptor
PSNL	Partial sciatic nerve ligation model of neuropathic pain (Seltzer model)
ROS	Reactive oxygen species
ROS	Reactive oxygen species
RVM	Rostral ventromedial medulla
sIL-1R1 / sIL-1R2 / sIL-1RAcP	Soluble IL-R1 / soluble IL-1R2 / soluble IL-1RAcP
SLC	Secondary lymphoid-tissue chemokine
SNI	Spared nerve injury model of neuropathic pain
SNT	Spinal nerve transection
SNT	Spinal nerve transection model of neuropathic pain
SP	Substance P
STAT	Signal transducer and activator of transcription
TAK-1	Transforming growth factor β -activated kinase-1
TIR domain	Toll / IL-1R domain
TLR	Toll-like receptor
TMP	Thiamine monophosphatase
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor-associated factor
Trk-A	Neurotrophic tyrosine kinase receptor - A
TRP	Transient receptor potential
TRX	Thioredoxin
TTX	Tetrodotoxin
TXNIP	Thioredoxin-interacting protein
VIP	Vasoactive intestinal peptide
WDR	Wide dynamic range

General Introduction

1. *Chronic pain*

Pain is an unpleasant and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP Pain Terminology). Thus pain is a protective mechanism, serving to warn the individual of tissue damage and any associated danger. Because it is an unpleasant experience, pain should elicit a behavioural response such as withdrawal from the harmful stimulus and some form of learning response to prevent further damage from such stimuli. The fundamental importance and adaptive function of such a warning system is highlighted in the unique cases of individuals with the rare phenotype of a complete inability to sense pain, resulting in inadvertent self-mutilation and repeated injuries from a young age (Cox et al., 2006).

Nociceptive, or acute pain, occurs following stimulation of the high-threshold nociceptors in the periphery. This pain is short-lasting and should cease upon withdrawal from the noxious stimulus and tissue healing. Chronic pain may be defined as pain which persists for longer than three months. This duration is relatively arbitrary, and perhaps a better definition would be pain that continues after resolution of the initial insult and the normal time of tissue healing. Treatment of chronic pain is difficult, with existing treatments only partially efficacious, seldom providing complete relief (Dworkin et al., 2010). Generally chronic pain is considered in three broad categories; inflammatory, neuropathic, and idiopathic, although these classifications are simple and imply distinct and separate mechanisms, when in reality the lines of distinction are more blurred with some overlapping mechanisms. Inflammatory pain is caused by the action of inflammatory mediators on nociceptive afferents. Inflammatory pain may occur acutely, but may also become chronic such as in osteoarthritis. However inflammatory pain generally resolves with resolution of the initiating inflammation. Neuropathic pain occurs as a consequence of lesion or disease of the somatosensory nervous system (Treede et al., 2008). There are four broad aetiologies of neuropathic pain; peripheral focal and multifocal nerve lesions (traumatic, ischaemic, or inflammatory), peripheral generalised polyneuropathies (toxic, metabolic, hereditary, or inflammatory), CNS lesions (eg stroke, multiple sclerosis, spinal cord injury), and complex

neuropathic disorders (complex regional pain syndromes (CRPS)) (Baron, 2006). Finally some chronic pain disorders must be described as idiopathic when no obvious pathological cause can be identified, such as in the case of fibromyalgia.

1.1. Neuropathic pain

Patients with neuropathic pain exhibit abnormal responses of the sensory nervous system and may present with positive or negative symptoms, or a combination of both. A decrease in stimulus intensity threshold to signal pain can result in positive symptoms such as hyperalgesia (an increased sensitivity to a noxious stimulus), allodynia (pain due to a normally non-noxious stimulus), as well as dysethesia (an unpleasant abnormal sensation, which may be spontaneous or evoked), or spontaneous (not stimulus-induced) pain. Patients may also experience negative symptoms such as a loss of sensation to mechanical and thermal stimuli, which may be accompanied by spontaneous pain in the region (anaesthesia dolorosa) (Baron, 2006). Although there are several different aetiologies of neuropathic pain, there is no simple link between the initial aetiology and the resultant symptoms experienced by the patient, and often there is overlap of symptoms. Additionally a lesion of the nervous system will not necessarily, and in many cases does not, result in neuropathic pain.

Treatment of neuropathic pain is difficult. Many patients have pain that is refractory to treatment, and for those that do report pharmacological relief it is often incomplete. Current pharmacotherapies for the management of neuropathic pain include tricyclic antidepressants (eg nortriptyline), selective serotonin and noradrenaline reuptake inhibitors (eg duloxetine, venlafaxine), gabapentinoids (gabapentin, pregabalin), topical lidocaine or capsaicin application, and opioids (eg tramadol) (Dworkin et al., 2010). This list of drugs represents a wide number of drug classes and actions, demonstrating the fact that there are a number of pathophysiological mechanisms contributing to the different pain symptoms reported. A recent and ongoing strategy for the treatment of neuropathic pain has been to group patients not by the aetiology of their pain, but rather by the classification of positive and negative symptoms

in response to different modalities of stimuli in order to better target treatment therapies (Baron et al., 2009).

1.2. *Inflammatory pain*

Inflammatory pain may be acute, for instance in the case of, for example, burns or penetration wounds. Common examples of chronic inflammatory pain include arthropathies such as gout, osteoarthritis and rheumatoid arthritis. In these conditions, the underlying initiating cause of pain is clear and lies in the pathology of the joint. However it is reported that there is often poor correlation between the clinical progression of the pathology and the occurrence of pain in these conditions (Schaible et al., 2009). Several treatments are available for chronic inflammatory pain, principally aimed at reducing or antagonising the action of inflammatory mediators such as IL-1 β or TNF- α (Schaible et al., 2010, Miller and Ranatunga, 2012).

1.3. *Animal models of pain*

Much of the difficulty in treating chronic pain lies in a lack of understanding of the underlying mechanisms. For many years now animal models have been developed and used to investigate the underlying mechanisms in neuropathic pain. The earliest models utilised involved traumatic lesions of a peripheral nerve; the chronic constriction injury model (CCI) (Bennett and Xie, 1988), the partial sciatic nerve ligation (PSNL), or Seltzer model (Seltzer et al., 1990), the L5 or L5/6 spinal nerve transection model (SNT) (Kim and Chung, 1992), and the spared nerve injury model (SNI) (Decosterd and Woolf, 2000). These models are detailed in Figure i.1, and Table i.1. The development of these models has been of great value in studying the development of underlying pathophysiological changes along multiple levels of the somatosensory signalling system following peripheral nerve lesion, as detailed in the next section, and their use continues to be widespread for this purpose. However, more recently concerns about the clinical relevance of such models has been raised, leading to the development of models based on metabolic, toxic, or infection-induced neuropathic pain models. These include streptozotocin-induced diabetic neuropathy (Malcangio and Tomlinson, 1998), varicella zoster virus-induced model of neuropathic pain (Fleetwood-Walker et al., 1999), HIV-

envelope protein glycoprotein 120-induced neuropathy (Wallace et al., 2007), bone cancer model of neuropathic pain (Schwei et al., 1999), and chemotherapy-induced models of neuropathic pain (Jaggi and Singh, 2012). Typically outcome measures in animal models of pain look at changes in sensitivity to evoked stimuli such as in the von Frey test for mechanical sensitivity, the Hargreaves and hot plate tests for heat sensitivity, and the acetone and cold plate tests for cold sensitivity. These tests aim to determine the presence or not of hyperalgesia and allodynia. However it must be remembered that they cannot account for spontaneous pain, and indeed may be confounded by the development of negative (loss of sensation) symptoms both commonly reported in neuropathic patients. Other behavioural tests such as the open field paradigm have been employed in an attempt to model different behavioural aspects of the pain experience.

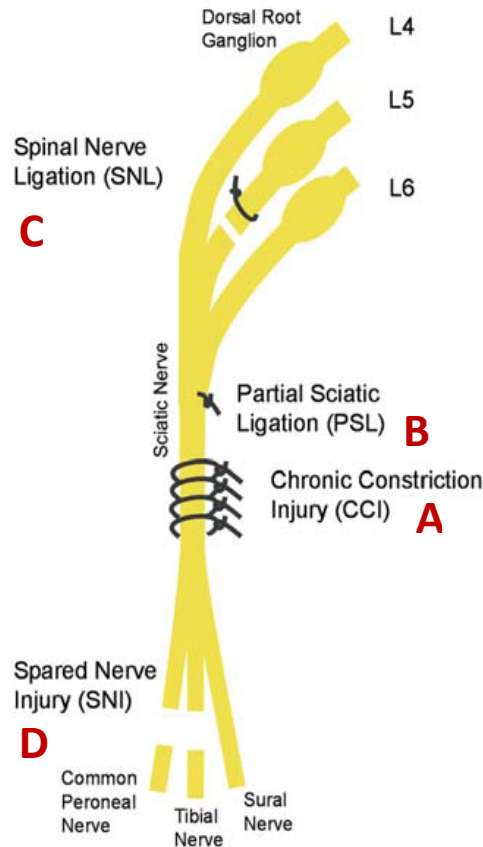


Figure i.1: Commonly used models of peripheral nerve injury

Schematic representing the methods of four commonly used animal models of peripheral nerve injury, as models of neuropathic pain. **a:** In the Chronic Constriction Injury model four loose chromic-gut ligatures are tied around the sciatic nerve at mid-thigh level. **b:** In the Partial Sciatic Nerve Ligation (PSNL), or Seltzer, model $\frac{1}{2}$ - $\frac{1}{3}$ of the sciatic nerve is tightly ligated at the high-thigh level. **c:** In the Spinal Nerve Ligation (SNL) / Spinal Nerve Transection (SNT), or Chung model the L5 or the L5 and L6 spinal nerves are exposed distal to the DRG by removal of the covering transverse process(es). The nerve(s) are then tightly ligated and may then be transected as in SNT. **d:** In the Spared Nerve Injury (SNI) model the common peroneal, and tibial nerves are sectioned, sparing the sural nerve. Figure adapted from (Campbell and Meyer, 2006).

Table i.1: Commonly used models of peripheral nerve injury

Model	Reference	Method	Comments
Chronic Constriction Injury (CCI) Model	(Bennett and Xie, 1988)	Four loose chromic-gut ligatures are tied around the sciatic nerve at mid-thigh level	This model has both an inflammatory and neuropathic component. An inflammatory reaction to the ligatures causes swelling, granuloma, and nerve swelling, which may ultimately damage fibres located at the surface of the nerve.
Partial Sciatic Nerve Ligation (PSL) / Seltzer Model	(Seltzer et al., 1990)	$\frac{1}{2}$ - $\frac{1}{3}$ of the sciatic nerve is tightly ligated at the high-thigh level	This model is more clinically relevant since not all of the axon is damaged. However there may be issues of reproducibility in relation to the extent of damage, particularly between surgeons.
Spinal Nerve Transection (SNT) / Spinal Nerve Ligation (SNL) / Chung Model	(Kim and Chung, 1992)	The L5 or the L5 and L6 spinal nerves are exposed distal to the DRG by removal of the covering transverse process(es). The nerve(s) are then tightly ligated and may then be transected as in SNT	The main advantage of this model over others is the ability to identify and study differences in axotomised primary sensory neurons (all those found in the L5 or L5/6 DRG), and neighbouring intact primary sensory neurons (all those found in the L4 DRG).
Spared Nerve Injury Model (SNI)	(Decosterd and Woolf, 2000)	The common peroneal, and tibial nerves are sectioned, sparing the sural nerve.	This model has an advantage over the Seltzer model in that the extent of injury should be less variable between surgeons.

There are several, commonly used, animal models of inflammatory pain. Acute and medium-term models often involve the administration of an inflammatory substance such as carrageenan or Complete Freund's Adjuvant (CFA). Longer term, or chronic, models of inflammatory pain include the intra-articular injection monosodium iodoacetate (MIA) or CFA into the knee joint to induce joint destruction as a model of osteoarthritis, or inflammatory mono-arthritis respectively. Animal models of rheumatoid arthritis are also commonly employed in which an immune response is induced by the administration of immunogens such as CFA or cartilage antigens (Williams, 2004, Alzabin and Williams, 2011, Barrot, 2012).

In animal models of both neuropathic and inflammatory pain there are several neurochemical changes occurring throughout the sensory neuraxis. In some cases there is overlap of these changes, and in others there are clear differences. Neurochemical changes following peripheral nerve injury in animal models of neuropathic pain are particularly well-characterised. The remainder of this section will discuss nociceptive signalling in the normal state, and the changes which occur in the injured or inflamed state. Subsequently the contribution of the immune response to chronic pain states will be discussed in Section 3.

2. Pain signalling and mechanisms of pain

The experience of pain is a complex one involving multiple levels of the nervous system, each capable of modulating the perceived sensation. Under normal non-pathological conditions the process of sensation begins in the periphery following stimulation. Sensation from the periphery is conveyed via the excitation of peripheral terminals of primary afferent fibres, whose cell bodies reside in the dorsal root and trigeminal ganglia and bifurcate to give rise to both the peripheral branch and a central branch terminating in the dorsal horn of the spinal cord. Based on their functional and structural properties, these are broadly classified into three main types; A β , A δ , and C fibres (Millan, 1999, D'Mello and Dickenson, 2008). A β fibres are the largest in diameter ($>1\text{ }\mu\text{m}$) and are thickly myelinated, resulting in a fast conduction velocity ($30\text{--}100\text{ms}^{-1}$). These fibres have a low threshold for activation and respond to mechanical stimuli making them ideal for the purpose of conveying light touch and tactile information. A δ fibres are smaller in diameter ($2\text{--}6\text{ }\mu\text{m}$), and are more thinly myelinated than A β fibres, thus have a slower conduction velocity ($12\text{--}20\text{ ms}^{-1}$). They respond to thermal and mechanical stimuli, and can respond to both innocuous and noxious inputs. C fibres have the smallest diameter ($0.1\text{--}1.2\text{ }\mu\text{m}$) and are unmyelinated, thus have a relatively slow conduction velocity ($0.5\text{--}2.0\text{ ms}^{-1}$). Generally they are able to respond to thermal, mechanical, and chemical stimulation. C fibres have a high threshold for activation meaning they predominantly encode for stimuli capable of causing tissue damage. It is generally thought that A δ fibres elicit the sharp first phase of pain felt upon noxious stimulation, whereas C fibres are responsible for the second dull-ache aspect of pain. Electrophysiological studies have enabled further subclassification of these primary afferent fibres based on responsiveness to different modalities of input, eg mechano-heat-responsive C fibres, C fibres responsive only to mechanical stimuli, C fibres responsive only to heat, and normally "silent" C fibres (Schmidt et al., 1995, Lawson, 2002).

Sensory modality of nociceptors, as well as their activation thresholds, is determined by their individual expressions of a repertoire of different receptors and ion channels. This is, in turn, determined early in development. The

eventual fate of a primary afferent fibre is determined by which growth factor receptor it expresses during development. Neurotrophic tyrosine kinase (Trk)-C-bearing fibres are responsive to the neurotrophin NT-3, and are predominantly large fibres giving rise to mainly large myelinated A β fibres in the mature animal. Trk-B-bearing fibres are responsive to the neurotrophin brain-derived neurotrophic factor (BDNF), and are predominantly small and medium-sized fibres. Trk-A-bearing fibres are responsive to the neurotrophin nerve growth factor (NGF), and are predominantly of a small diameter giving rise to C-fibres in the mature animal. In the rat and mouse neonate, approximately 80% of L4/5 dorsal root ganglion (DRG) neurons express TrkA. This proportion decreases to approximately 40% by the second postnatal week in mice, and the third postnatal week in rats. This decrease is the result of a switch in neurotrophin receptor expression from TrkA to Ret, the transmembrane signalling component of the receptor responsive to glial cell-derived growth factor (GDNF), resulting in two classes of C-fibre defined by their differing immunohistochemistry. The TrkA-expressing population develop into the peptidergic nociceptors, expressing calcitonin gene related peptide (CGRP) and Substance P (SP) and terminating in lamina I and II_{outer}. The Ret-expressing population develop into the non-peptidergic, isolectin B4 (IB4)-binding nociceptors, expressing also fluoride-resistant acid phosphatase (FRAP), thiamine monophosphatase (TMP) and purinergic ionotropic receptors P2X3 and terminating in lamina II_{inner} (Mu et al., 1993, McMahon et al., 1994, Molliver and Snider, 1997, Bradbury et al., 1998, Snider and McMahon, 1998, Stucky and Lewin, 1999, Hunt and Mantyh, 2001, Chen et al., 2006, Ernsberger, 2009). Intense mechanical, thermal or chemical stimuli are able to be transduced into electrical activity by the diverse array of ion channels expressed by nociceptors. Several ion channels and receptors are preferentially expressed in one of these two broad populations of nociceptors, their expression driven by specific neurotrophins.

2.1. *Peripheral sensitisation*

Although there is still much to learn, it is clear from the structured development, differentiation, and organisation of nociceptor expression and

synaptic termination that the process of sensation is a highly complex and ordered one. An important finding from the use of animal models of pain, such as those previously described, is that, following peripheral nerve injury or inflammation, this ordered system shows several plastic changes. Following tissue or peripheral nerve damage there is a change in the local chemical environment which can result in peripheral sensitisation of the primary afferents resulting in a reduction in threshold for activation and increase in responsiveness to stimulation. The change in the chemical milieu occurs as a result of the release of a multitude of mediators including neurotransmitters (eg 5-hydroxytryptamine (5-HT), histamine), peptides (eg SP, CGRP, bradykinin), cytokines and chemokines (eg interleukin (IL)-1 β , tumour necrosis factor (TNF)- α , chemokine (C-C motif) ligand 3 (CCL3)), protons, proteases, and neurotrophins (eg NGF), collectively referred to as an “inflammatory soup”, released from nociceptors as well as non-neuronal cells recruited to, or residing in, the local environment such as Schwann cells and macrophages. These inflammatory mediators act on their receptors expressed on nociceptor terminals, and exert their sensitising effects via second messenger action (eg protein kinase C (PKC), protein kinase A (PKA), phosphoinositol-3-kinase (PI3K), jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK)) on receptors and ion channels present in the nociceptor, most notably transient receptor potential (TRP) and voltage-gated sodium channels. Post-translational modification of these channels results in an alteration of their kinetics and thresholds of activation, resulting in an increase in sensitivity and excitability of the nociceptor terminal (Chuang et al., 2001, Wood et al., 2004, Pezet and McMahon, 2006, Hucho and Levine, 2007, Woolf and Ma, 2007, Basbaum et al., 2009, Uceyler et al., 2009). It had previously been thought that peripheral sensitisation of the nociceptors was of relevance only in inflammatory pain states, but it is now increasingly recognised to contribute to the initiation of neuropathic pain states also (Campbell and Meyer, 2006, Moalem and Tracey, 2006).

In addition to these short-term effects of “inflammatory soup” following damage or injury, there can also be longer-term consequences following second

messenger activation in the activation of transcription factors such as cAMP responsive element binding protein (CREB), signal transducer and activator of transcription (STAT), and activating transcription factor (ATF)-3. Changes in transcription factor activity in turn lead to characteristic changes in the expression of neurotransmitters, peptides, and ion channel proteins (Navarro et al., 2007). After peripheral nerve injury, mRNA and translated protein levels of SP, CGRP, and somatostatin have been shown to decrease in small nociceptors, whereas galanin and vasoactive intestinal polypeptide (VIP) levels are increased (Noguchi et al., 1990, Noguchi et al., 1994, Navarro et al., 2007). However in animal models of inflammatory pain SP and CGRP levels increase in the lumbar dorsal horn (Honor et al., 1999, Honore et al., 2000, Allen et al., 2003). Damage to peripheral nerves also induces upregulation of various receptor proteins. After peripheral nerve injury TRPV1 expression has been shown to decrease in damaged afferents, but increase in uninjured C- and A-fibres (Hudson et al., 2001, Ma et al., 2005). Larger DRG neurons undergo a phenotypic switch after peripheral nerve axotomy with the *de novo* expression of SP, BDNF, and TRPV1 usually expressed only in small nociceptive fibres (Noguchi et al., 1994, Zhou et al., 1999, Malcangio et al., 2000). Similarly in animal models of inflammatory pain TRPV1 expression increases in C fibres and there is *de novo* expression of SP in A β fibres (Neumann et al., 1996, Ji et al., 2002, Amaya et al., 2004). The non-inactivating tetrodotoxin (TTX) sensitive sodium channel Na_v1.3, usually expressed only during development, is upregulated in sensory neurons following peripheral axotomy, whereas Na_v1.1, Na_v1.2, Na_v1.6, Na_v1.7, Na_v1.8, and Na_v1.9 are all downregulated (Dib-Hajj et al., 1999, Waxman et al., 2000, Leffler et al., 2002). In animal models of inflammatory pain the changes in expression of voltage gated sodium channels differ, with increases in Na_v1.3, Na_v1.7, Na_v1.8, and Na_v1.9 reported (Tanaka et al., 1998, Tate et al., 1998, Black et al., 2004). Hyperpolarisation-activated cyclic nucleotide-modulated 2 (HCN2) ion channels, responsible for generating inward current to depolarise the membrane to threshold for subsequent action potential generation have been shown to be upregulated following peripheral nerve injury, and pharmacological blockade reduces primary afferent ectopic activity as well as behavioural hypersensitivity (Lee et al., 2005, Emery et al., 2011, Emery et al.,

2012). A-type potassium channels $K_v3.4$ and $K_v4.3$, crucial in controlling neuronal excitability, are also downregulated in sensory neurons after peripheral nerve injury (Chien et al., 2007). Similarly, in animal models of inflammation a suppression of A-type potassium currents has been observed, resulting in an overall enhancement of neuronal excitability (Takeda et al., 2006, Takeda et al., 2008).

As well as these changes in expression pattern of ion channels axotomy leads to a clustering of synthesised channels, normally transported along the axon in vesicles to be inserted at the non-myelinated nodes of Ranvier, inserted at the unmyelinated neuroma stump in damaged afferents (Dugandzija-Novakovic et al., 1995, Novakovic et al., 1998, Kretschmer et al., 2002, Devor, 2006). Such changes lead ultimately to an overall change in the excitability properties of sensory neurons, including reduced threshold for activation (Ma et al., 2003, Devor, 2006, Navarro et al., 2007). Indeed many studies have shown that there is development of spontaneous activity in injured afferents following peripheral nerve injury in animal models of pain, as well as in humans (Campbell et al., 1988, Boucher et al., 2000, Liu et al., 2000b). Interestingly these studies have emphasised the origin of spontaneous activity to be myelinated A β -fibres, with no spontaneous activity found in damaged C-fibres. Spontaneous activity has also been shown to develop in uninjured unmyelinated nociceptive afferents sharing the same innervation territory as the damaged fibres, and behavioural studies suggest that the development of spontaneous activity in these uninjured afferents contributes to the maintenance of neuropathic pain-like behaviours (Boucher et al., 2000, Wu et al., 2002, Ma et al., 2003, Djouhri et al., 2006). The L5 spinal nerve transection (SNT) model has been of great use in studying these changes as all the L4 DRG neurons are left intact, whereas all L5 DRG neurons are damaged. Using this model other changes have been reported in the intact L4 DRG, including increased expression of TRPV1, TRPA1, and CGRP mRNA (Boucher and McMahon, 2001, Campbell and Meyer, 2006). It is postulated that the reasons for changes in uninjured afferents stems from a change in target-derived neurotrophic factors as a result of the lesion of the injured afferents innervating the same territory. Here it is thought that release of NGF at the site of injury

from denervated Schwann cells binds to Trk-A receptors on the uninjured afferents to bring about trophic changes. In addition to this increased presence of NGF, its availability is, too, increased since there is reduced competition for uptake from the injured afferents. Many of the changes following peripheral nerve axotomy are likely due to a change in neuronal drive to promote a state of survival and repair, and are largely brought about by changes in neurotrophin levels both at the target and synthesised by the damaged neuron. Interestingly intrathecal delivery of different neurotrophins is able to variously reverse or attenuate many of these changes (Bennett et al., 1996, Bennett et al., 1998, Boucher et al., 2000, Boucher and McMahon, 2001, Pezet and McMahon, 2006). The development of spontaneous activity in sensory neurons is a feature of animal models of inflammation also. Following intraplantar injection of CFA spontaneous activity develops in both A- and C-fibres, and is correlated with the development of behavioural hypersensitivity (Djoughri et al., 2006, Xiao and Bennett, 2007).

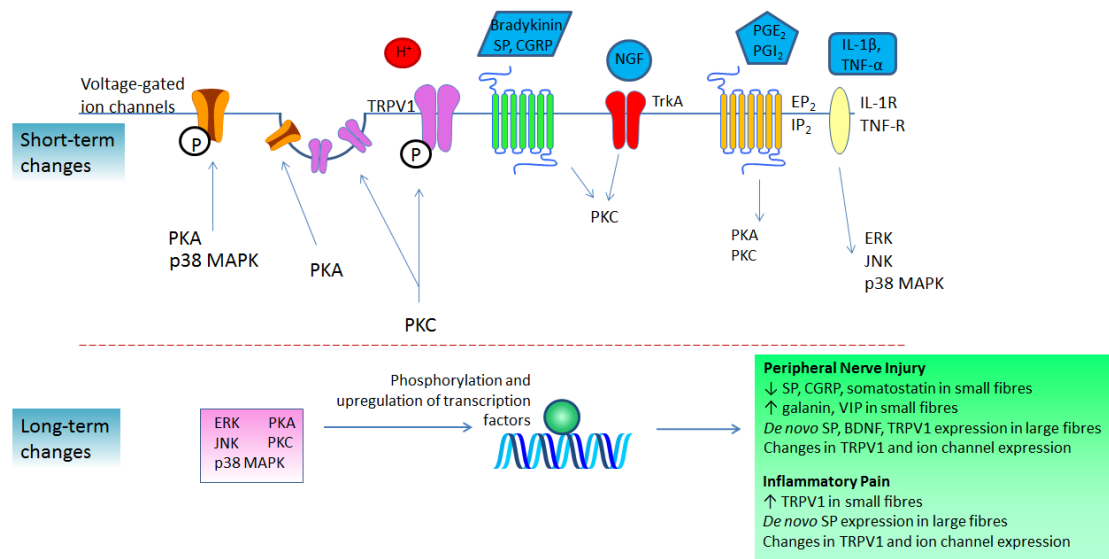


Figure i.2: Mechanisms of peripheral sensitisation of sensory neurons

Schematic representing mechanisms of peripheral sensitisation in animal models of pain. Peripheral nerve damage or inflammation causes resident and infiltrating immune cells, as well as the sensory nerve terminals themselves, to release inflammatory mediators including IL-1 β , TNF- α , bradykinin, SP, CGRP, NGF, and prostaglandins, contributing to the “inflammatory soup”. Inflammatory mediators act on their receptors on primary afferents and sensitise sensory neurons via second messenger (eg PKA, PKC, ERK, JNK, p38 MAPK) actions. PKC action increases TRPV1 insertion into the plasma membrane, and phosphorylates TRPV1 receptors decreasing activation thresholds. PKA and p38 MAPK activation lead to increased insertion of voltage-gated sodium channels into the plasma membrane, and phosphorylation of ion channels, decreasing activation thresholds. In the long-term second messenger activation leads to the phosphorylation and upregulation of transcription factors leading to changes in neurotransmitter, receptor, and ion channel expression.

2.2. Nociceptive processing in the dorsal horn

The central terminal of primary afferent neurons synapse onto second order neurons in the dorsal horn of the spinal cord in a specific distribution pattern, determined by their functional class. Generally high-threshold nociceptive afferents terminate superficially in laminae I and II, whereas the low threshold myelinated mechanoresponsive afferents terminate predominantly in laminae III – V. C-fibres terminate differentially depending on their neurochemical class, whereby peptidergic C-fibres terminate in laminae I and II_{outer}, and non-peptidergic C-fibres terminate in lamina II_{inner}. Broadly speaking, there are three types of second-order neuron within the dorsal horn responsive to primary afferent sensory information. These are non-nociceptive, nociceptive, and wide dynamic range (WDR) neurons. Non-nociceptive neurons, found primarily in laminae III and IV, respond only to innocuous tactile information coming from A β primary afferent input. Nociceptive-specific neurons are found more superficially in laminae I and II, receiving noxious input from nociceptive A δ - and C-fibres. WDR neurons are located predominantly in lamina V and receive, indirectly, input from all three types of primary afferent fibre. Thus they are able to encode sensory information over the range of modalities and intensities, displaying stimulus intensity-dependent responses (Millan, 1999, D'Mello and Dickenson, 2008, Todd, 2010). Following peripheral nerve injury it has been postulated that, in addition to phenotype switching of myelinated fibres, there is a sprouting of the central terminals of A β fibres into superficial laminae allowing access to nociceptive pathways (Woolf et al., 1992, Kohama et al., 2000, Watanabe et al., 2007). This, along with the change in neurotransmitter expression of these fibres, may possibly explain why normally innocuous, low-threshold stimuli, can become painful in some patients. However, it remains controversial as to whether these changes do occur following reports that some of the labelling techniques employed in these studies lack specificity and the recent finding that, in the thoracic cord of the mouse, A β -fibres in fact projected throughout the dorsal horn laminae I-V (Tong et al., 1999, Hughes et al., 2003, Shehab et al., 2003, Woodbury et al., 2008).

Input from the periphery is signalled by the release of glutamate onto second order neurons within the dorsal horn. Under non-pathological conditions signalling is primarily through activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or kainate receptor subtypes. N-methyl-D-aspartate (NMDA) receptors are also present, but the presence of a Mg^{2+} block at the ion channel pore prevents flow of current. Sustained slow depolarisation of the membrane induced by the co-release of peptidergic transmitters such as SP and CGRP during continuous or repetitive input from the periphery is necessary for the release of this block. Subsequent NMDA receptor-mediated increases in calcium influx leads to downstream activation of signalling pathways and second messenger action which are able to modulate post-synaptic receptor function, for instance via phosphorylation of NMDA receptors and increased trafficking of AMPA receptors to the surface. The *de novo* expression of SP by large myelinated A β afferent fibres following peripheral nerve injury or inflammation may be one mechanism by which low-threshold stimulation begins to generate painful sensations in patients with allodynia (Campbell and Meyer, 2006, D'Mello and Dickenson, 2008, Costigan et al., 2009b). Vesicular release of neurotransmitters from the central terminals of primary afferent fibres is triggered by voltage-gated calcium channel activity. Following peripheral nerve injury it has been shown that the $\alpha_2\delta-1$ calcium channel subunit is upregulated in both DRG and the dorsal horn of the spinal cord (Li et al., 2004, Bauer et al., 2009). This may lead to increased neurotransmitter release following peripheral input, and thus may represent another mechanism of central sensitisation following peripheral nerve injury.

Input from the periphery is subject to substantial modulation within the dorsal horn from interneurons, both excitatory and inhibitory. One way in which sensitisation appears to occur following peripheral nerve injury is via the disinhibition of peripheral afferent signalling. Pre-synaptic release of glutamate from primary afferents can be inhibited by activation of pre-synaptic μ -opioid receptors. It has been shown that expression of μ -opioid receptors is decreased both pre- and post-synaptically following peripheral nerve injury, presumably leading to an increase in excitatory transmission from the periphery. The K^+Cl^- transporter KCC2 is responsible for maintaining K^+ and Cl^-

gradients across the neuronal membrane. It has been shown that this transporter, normally expressed in lamina I projection neurons, is downregulated in an animal model of neuropathic pain and inflammatory pain. As a result there is a shift in the anion gradient across the neuronal membrane such that gamma-aminobutyric acid (GABA) action, which is normally hyperpolarising, causes neuronal depolarisation instead (Coull et al., 2003, Zhang et al., 2008b). There is evidence to suggest a microglial contribution to this phenomenon (Coull et al., 2005), which will be further discussed in Section 4.1.2. In addition to this change in GABA action, it has also been reported that GABA transmission is reduced following peripheral nerve injury. A reduction in levels of glutamic acid decarboxylase (GAD) in lamina II of the dorsal horn has been shown following peripheral nerve injury in several animal models of neuropathic pain (Moore et al., 2002, Scholz et al., 2005). This has been attributed to neuronal apoptosis within the dorsal horn, although there are conflicting reports (Polgar et al., 2005). The expression of the gamma isoform of protein kinase C (PKC γ) is restricted to lamina II_{inner}, and is likely to represent a population of excitatory interneurons (Polgar et al., 1999). Transgenic mice lacking PKC γ have been shown to display normal responses to acute noxious stimuli, but fail to develop thermal and mechanical hypersensitivity after peripheral nerve injury (Malmberg et al., 1997). It has more recently been shown that PKC γ expressing neurons receive input from large myelinated A β -fibres, and are normally subject to inhibitory glycinergic modulation (Miraucourt et al., 2007, Neumann et al., 2008). Peripheral nerve injury causes ectopic activity in A β -fibres, as well as a decrease in glycinergic inhibition (Miraucourt et al., 2007), both of which may contribute to the generation of painful sensation to normally innocuous low-threshold mechanical stimuli.

In animal models of inflammatory pain there is also a disinhibition of glycinergic transmission, primarily in the superficial dorsal horn. Spinal cord increases in PGE₂ following a peripheral inflammatory stimulus have been shown to inhibit glycinergic neurotransmission via PKA second messenger action (Beiche et al., 1996, Ahmadi et al., 2002). Interestingly PKA has been shown to have a greater contributory role in inflammatory pain, whereas PKC has been shown

to be of greater importance in the development of neuropathic pain (Yajima et al., 2003). This finding is in agreement with the finding that mice deficient in PKA showed reduced behavioural hypersensitivity in a model of inflammatory pain, but developed peripheral nerve injury-induced behavioural hypersensitivity normally (Malmberg et al., 1997).

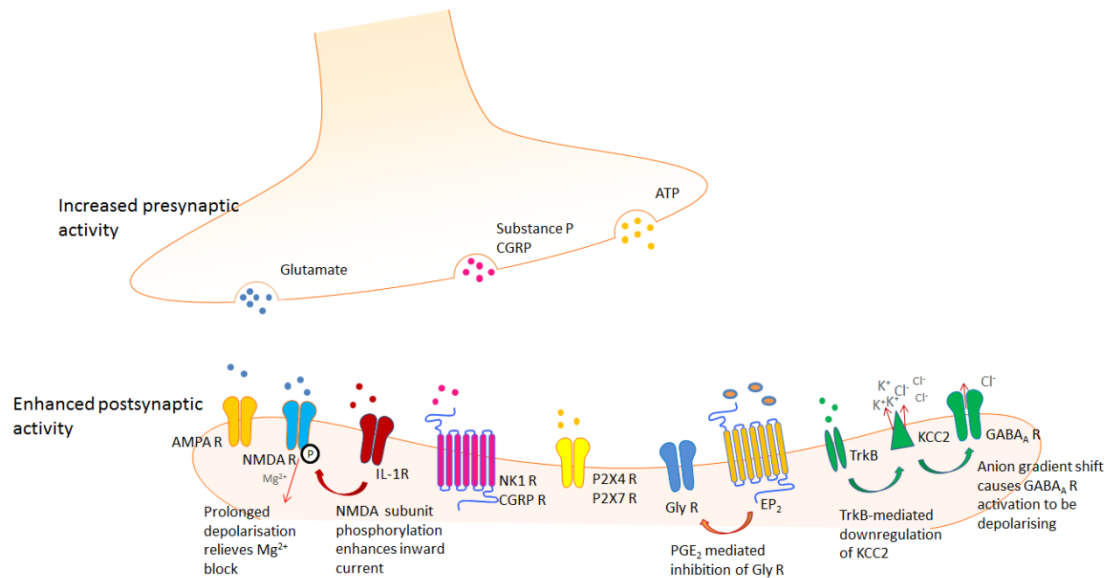


Figure i.3: Mechanisms of dorsal horn sensitisation

Schematic representing mechanisms of dorsal horn sensitisation in animal models of pain. Enhanced pre-synaptic activity leads to prolonged post-synaptic depolarisation via glutamatergic action at AMPA receptors, and SP and CGRP action at metabotropic receptors. Prolonged depolarisation of the membrane relieves the physiological Mg²⁺ block of NMDA receptors, with subsequent activation further enhancing postsynaptic activity and leading to second messenger-mediated changes. ATP released from pre-synaptic terminals acts on post-synaptic P2X4 and P2X7 receptors. In peripheral nerve injury models of neuropathic pain TrkB-mediated downregulation of K⁺Cl⁻ transporter KCC2 results in an anion gradient shift, causing GABA_A receptor activation to be depolarising. Evidence for a microglial contribution to this phenomenon is discussed in section 4.1.2. In animal models of inflammatory pain disinhibition of the post-synaptic terminal is brought about by PGE₂-mediated downregulation of glycine receptors.

2.3. Brainstem modulation

The eventual output from signalling from the dorsal horn to higher centres in the brain is via ascending pathways signalled by spinal projection neurons. These neurons are found predominantly in lamina I, though there are also projection neurons to be found throughout lamina III-VI. Using antero- and retrograde tracing techniques it has been shown that the main targets of lamina I projection neurons are the caudal ventrolateral medulla, the nucleus of the solitary tract, the lateral parabrachial area, the periaqueductal grey matter (PAG), and certain nuclei within the thalamus and these areas may be subject to modulation from the limbic system (Todd, 2010). Around 80% of lamina I projection neurons have been shown to express the neurokinin-1 (NK-1) receptor, which binds the peptide Substance P, and thus have been suggested to be nociceptive-specific. In line with this view, specific ablation of NK-1 expressing spinal neurons via the administration of a SP-conjugated toxin prevents the development of hyperalgesia in response to Intraplantar capsaicin treatment, without affecting baseline responses (Mantyh et al., 1997). Projection neurons from deeper laminae III – VI project predominantly to the thalamus which has further connections to cortical regions such as somatosensory cortices, the insular, the anterior cingulate, and the prefrontal cortices, collectively often referred to as the “pain matrix” (Tracey and Mantyh, 2007, Todd, 2010). The pain experience does not stop at these higher centres, however, and sensory processing within the spinal cord is subject to further modulation from higher centres via descending pathways from brainstem structures, principally the rostral ventromedial medulla (RVM) and periaqueductal grey (PAG), projecting heavily to the superficial dorsal horn (D'Mello and Dickenson, 2008, Heinricher et al., 2009). Early studies identified the PAG as a promising target for pain modulation when it was shown that electrical stimulation of the PAG resulted in analgesia, fuelling much research into the field of descending modulation of pain pathways. The PAG does not project directly to the spinal cord, however, instead projecting first to the RVM.

It is now well-appreciated that descending control of sensory pathways is bidirectional, with both facilitatory and inhibitory drives. Characterisation of neuronal cell types in the RVM led to the identification of three cell types;

“ON-cells”, “OFF-cells”, and “NEUTRAL-cells” (Fields et al., 1983). The tails of lightly anaesthetised rats were exposed to heat, generating a tail-flick response. Neuronal discharges recorded from ON-cells were found to increase immediately prior to the tail-flick, whereas neuronal discharges recorded from OFF-cells were found to decrease at this time. The NEUTRAL-cells were so-called since their activity remained unchanged. Since this time many other reports have confirmed this characterisation. As a result it is generally believed that ON-cells mediate descending facilitation, and OFF-cells mediate descending inhibition (Vanegas and Schaible, 2004). Further work has suggested that under normal conditions, as well as after nerve injury and inflammation, the net balance of output of descending pathways is facilitatory since injection of lignocaine into the RVM causes a reduction in deep dorsal horn neuronal responses to electrical and natural stimuli (Porreca et al., 2002). This effect was greater in animals that had received a peripheral nerve injury, and further included responses to non-noxious stimuli (Bee and Dickenson, 2007). The facilitatory transmitter in this pathway is 5-HT, which acts on spinal 5-HT₃ receptors located on nerve terminals of small diameter afferents (Millan, 2002, Suzuki et al., 2002, Zeitz et al., 2002). Under normal, non-pathological, conditions there is also a tonic inhibitory drive from higher centres, mediated by noradrenaline acting at α 2-adrenoreceptors, and following peripheral nerve injury this drive is reduced (Millan, 2002, Rahman et al., 2008) further shifting the balance of descending modulation to that of facilitation of nociceptive signalling from the periphery.

3. *The immune response in pain*

Inflammation is the process by which an organism responds to tissue injury and is an attempt to remove the injurious stimuli and to initiate healing. This constitutes immune cell recruitment and mediator release in response to diverse forms of tissue injury of which pathogen infection is only one component. The inflammatory response consists of a pro-inflammatory phase in which any pathogens are removed, damaged cells and debris cleared, and the local homeostasis restored. Following this there is a phase of resolution in which there is local tissue repair and the potentially damaging effects of a continued inflammatory response are dissipated. Several cell types mediate the immune response with temporally distinct contributions. The immune response is recruited and mediated via the release of a range of chemical mediators, many of which have effects in common. Inflammation is a well-established cause of nociceptive pain whether due to autoimmunity such as in rheumatoid arthritis or chemical mediators such as in gout. In addition many diverse aetiologies of neuropathic pain such as traumatic neuropathy or spinal cord injury are associated with excessive inflammation. The following section will discuss inflammatory mechanisms at differing levels of the sensory neuraxis which play a role in persistent pain states. Although the inflammatory response is likely to differ depending on the physiological situation, an overview of the stages of an inflammatory response is illustrated in Figure i.4.

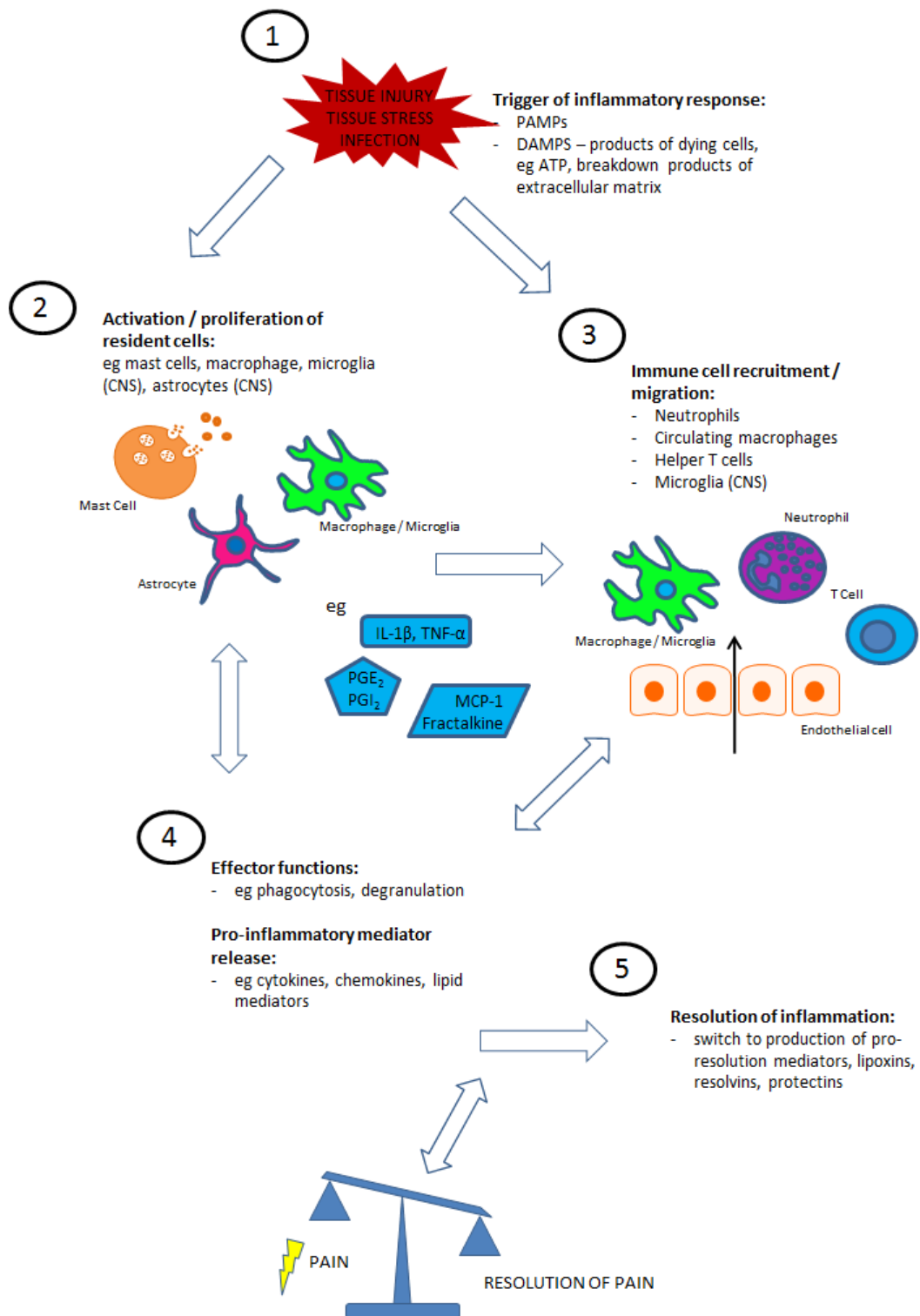


Figure i.4: (figure legend on the next page)

Figure i.4: Overview of the stages of the inflammatory response

Schematic representing the stages of the inflammatory response. **1:** Infection, tissue injury, or stress can trigger the immune response via activation of receptors responsive to pathogen-associated molecular patterns (PAMP), such as LPS, and danger-associated molecular patterns (DAMP), such as uric acid. **2:** This leads to the activation, migration, and proliferation of resident immune cells, including mast cells and macrophages. **3:** Activated immune cells release inflammatory mediators which mediate **4:** recruitment of circulating immune cells, including neutrophils, macrophages, and T cells. Inflammatory mediator release from these, and resident, cells also induces **4:** immune cell effector functions such as phagocytosis, and brings about further inflammatory mediator release. Pro-inflammatory mediators excite sensory afferents generating pain signals. **5:** As the course of the inflammatory process progresses, there is a switch in mediator production to pro-resolution mediators such as resolvins. These promote resolution of the inflammatory response, limiting further immune cell infiltration and promoting phagocytosis of infiltrated immune cells. There is evidence that such resolution of inflammation can also reduce pain sensitivity.

3.1. The inflammatory response within the peripheral nerve trunk to injury

For many years research into pain signalling, and pathological pain states, focused on the role of neuronal changes and activity as discussed in the previous section. However it is now well-accepted that there is a key role for the immune system in the initiation and maintenance of pathological pain states. As previously mentioned damage to peripheral nerves generates an inflammatory response at the site of injury, mediated by immune cells recruited to the site of injury to remove cellular debris of degenerating axons (Wallerian degeneration) and facilitate regeneration. The first cells to react to damage of the nerve are resident immune cells; Schwann cells, mast cells, and macrophages. Myelinating Schwann cells dedifferentiate and begin the process of degrading the myelin sheath at the site of injury, a necessary prerequisite for regeneration, prior to macrophage recruitment (Allodi et al., 2012). An as yet unspecified signal results in the activation of the ERK MAP kinase signalling pathway in these cells and this is one of the earliest events triggering the expression of inflammatory mediators and recruiting immune cells to the damaged nerve (Napoli et al., 2012). TNF- α secreted by Schwann cells, and also resident mast cells following nerve injury, induces the production of MMP-9 which degrades myelin basic protein and also promotes macrophage recruitment (Wagner and Myers, 1996, Kobayashi et al., 2008a). Additionally TNF- α secreted by Schwann cells induces the production of monocyte chemoattractant protein-1 (MCP-1) and leukotriene inhibitory factor (LIF) which also promote macrophage recruitment (Subang and Richardson, 2001, Tofaris et al., 2002, Shubayev et al., 2006). After injury resident mast cells degranulate releasing inflammatory mediators including histamine, 5-HT, nerve growth factor (NGF), and leukotrienes, which can sensitise nociceptors and also contribute to the recruitment of neutrophils, the first cells to infiltrate damaged tissue (Perkins and Tracey, 2000, Zuo et al., 2003, Smith et al., 2007, Kim and Moalem-Taylor, 2011). Mast cell stabilisation with sodium cromoglycate reduces the infiltration of neutrophils to the injured nerve and suppresses the development of peripheral nerve injury-induced thermal and mechanical hypersensitivity, highlighting the importance of the early immune response in the development of neuropathic pain (Zuo et al., 2003). Similarly,

using an anti-neutrophil antibody to deplete circulating neutrophils at the time of injury has been shown to significantly attenuate subsequent behavioural hypersensitivity (Perkins and Tracey, 2000). Neutrophil infiltration to the site of injury is acute, peaking within the first few hours after injury and declining after three days, though levels remain elevated. Neutrophils also release mediators capable of sensitising nociceptors, as well as recruiting macrophages and T cells to the injury site (Moalem et al., 2004, Kumar and Sharma, 2010). T cells are characterised by the expression of surface molecule CD4 (CD4+ T helper (Th) cells) or CD8 (CD8+ cytotoxic T cells), and are further subclassified as Type 1 or 2 based on their cytokine expression profile. Infiltrating T cells release pro-inflammatory cytokines such as IL-1 β , TNF- α , in the case of Th1 cells, and IL-17, in the case of Th17 cells. However Th2 cells release mainly anti-inflammatory cytokines such as IL-4 and IL-10 (Palmer and Weaver, 2010). Recruited infiltrating macrophages join resident cells and, along with Schwann cells, take part in the phagocytosis of degenerating axons and myelin sheaths. In addition to this function, they are capable of secreting a myriad of substances including prostaglandins E₂ and I₂, cytokines including IL-1 β , IL-6, IL-8, and TNF- α , and the leukotriene LTB₄ (Nathan, 1987). Depletion of the circulating population of macrophages via the administration of liposomes containing clodronate reduces behavioural hypersensitivity following peripheral nerve injury (Liu et al., 2000a). Perhaps unsurprisingly, a role for neutrophils, mast cells, macrophages, and T cells has been shown in animal models of inflammatory pain also (Woolf et al., 1996, Kidd and Urban, 2001, Tanaka et al., 2006, Ulmann et al., 2010, Alzabin and Williams, 2011, Boue et al., 2011, Li et al., 2012).

In addition to the release of inflammatory mediators from infiltrating immune cells, the sensory terminals release neuropeptides such as Substance P and CGRP upon antidromic conduction of impulses. These vasoactive peptides may enhance the immune response by their actions increasing vascular permeability and may actually directly interact with immune cells such as Langerhan's cells and macrophages. Additionally they act directly on primary afferent neurons, further stimulating and sensitising the sensory pathway (Torii et al., 1997, Gonzalez et al., 2005). The change in the local chemical milieu

brought about by the inflammatory response not only has actions on damaged, degenerating, neurons, but also on the neighbouring uninjured afferents sharing the same innervation territory. As a result these fibres exhibit spontaneous activity (Wu et al., 2002, Ma et al., 2003). Spontaneous activity also develops following peripheral administration of an inflammatory stimulus, such as CFA (Djouhri et al., 2006, Xiao and Bennett, 2007). The sensitising action of mediators of the inflammatory response can be due to a direct action on nociceptors, in the case of PGE₂ and PGI₂, or an indirect action. IL-1 β indirectly sensitises nociceptors as its release leads to prostaglandin synthesis, and it is also able to directly sensitise nociceptors via actions on TRPV1 receptors (Kiguchi et al., 2012).

The injured DRG, and to a lesser extent adjacent DRG containing uninjured neurons innervating the injured territories, are also subject to an immune response following peripheral nerve injury or inflammation. Neutrophils have been shown to invade the ipsilateral L3-5 DRG following partial sciatic nerve ligation in the mouse at day 14, much later than that observed peripherally and at lower levels (Kim and Moalem-Taylor, 2011). Peripheral nerve injury also induces significant T cell infiltration, most of which are CD8+ as opposed to CD4+, to the ipsilateral DRGs from around 7 days after injury (Hu and McLachlan, 2002, Hu et al., 2007, Kim and Moalem-Taylor, 2011). Macrophages infiltrate ipsilateral DRGs, forming perineuronal rings around medium to large diameter neurons, from approximately 3 days post-injury, peaking at 7 days, and continuing for several weeks (Hu and McLachlan, 2002, Kim and Moalem-Taylor, 2011). Similarly macrophage infiltration of the DRG is reported in animal models of arthritis (Inglis et al., 2005, Segond von Banchet et al., 2009).

3.2. Central immune response

Unlike the peripheral nerve and DRG, the spinal cord is protected by the blood-spinal cord barrier, which was thought to prevent the same influx of immune cells following nerve injury and inflammation. However emerging reports suggest that peripheral nerve injury can result in a disruption of the blood-spinal cord barrier allowing influx of peripheral immune cells, an effect shown to be mediated by the action of MCP-1 (Beggs et al., 2010, Echeverry et al., 2011). In several animal models of pain a central immune response is reported, generally being strongest in surgical models of traumatic nerve injury where there is a profound immune response, mainly involving cell types resident to the CNS; astrocytes and microglia. There is also a low level infiltration of T cells into the dorsal spinal cord following peripheral nerve injury by leukocyte extravasation. This is likely to contribute to the neuropathic pain phenotype since mice deficient in T cells show attenuated behavioural hypersensitivity in response to peripheral nerve injury (Cao and DeLeo, 2008, Costigan et al., 2009a).

Glial cells constitute some 70% of the total cell population of the brain and spinal cord (Vallejo et al., 2010), with microglia comprising some 5% to 10% of the glial population, though for many years it was thought the role of CNS glia was simply one of neurotrophic support and immune protection. It is now well-established, however, that glia play an important role in the generation of persistent pain syndromes. Much research in recent years has focused, in particular, on the contribution of microglia to such pain states. The response of spinal cord microglia in a variety of animal models of pain has been investigated, particularly in nerve-injury induced pain models (Colburn et al., 1999, Hains and Waxman, 2006, Wen et al., 2007, Scholz et al., 2008, Hathway et al., 2009, Wodarski et al., 2009, Staniland et al., 2010, Clark et al., 2012). The microglial response to several commonly used nerve injury models, SNI, CCI, and SNL, was recently compared by Scholz *et al.*. The group found consistently an increase in CD11b (OX42 / complement receptor 3) immunoreactivity, a commonly used marker of microglial activation, in the spinal cord 3 days after injury of all types tested. This was maximal after 7 days, and had begun to decrease again by 14 days post-injury. This time

course was in agreement with several other studies (Jin et al., 2003, Wen et al., 2007, Tsuda et al., 2009b). Blockade of the microglial response with minocycline, a second-generation tetracycline, prevents the development of injury-induced behavioural hypersensitivity in rats (Raghavendra et al., 2003, Ledeboer et al., 2005), further implicating microglia in the development of hypersensitivity in animal models of pain. That minocycline is effective only in preventing the development of such behaviours, and is unable to reverse established hypersensitivity, suggests that microglia contribute only in the early, initiation, stages of pathological pain. In addition to animal models of traumatic peripheral nerve injury, microgliosis has also been observed in several other animal models of pain, including the formalin model, streptozotocin-induced diabetic neuropathy, collagen-induced arthritis, and bone cancer pain (Fu et al., 2009, Talbot et al., 2010, Clark et al., 2012, Hu et al., 2012). However the extent of this microglial response differs among models, generally being strongest in surgical models of traumatic nerve injury (Zheng et al., 2011, Blackbeard et al., 2012). And although a central glial response is observed in some animal models of pain, it is almost absent in others. For example there is little to no microgliosis observed in anti-retroviral 2', 3'-dideoxycytidine (ddC)-induced neuropathy or in the d4T-induced HIV-SIN model of neuropathic pain (Zheng et al., 2011, Huang, 2013). There are conflicting reports regarding the microglial response to chemotherapy-induced neuropathy, however (Sweitzer et al., 2006, Kiguchi et al., 2008, Zheng et al., 2011). In the streptozotocin-induced diabetic neuropathy model of neuropathic pain a small, but significant, increase in IBA1 is observed (Wodarski et al., 2009, Talbot et al., 2010). However in the db/db mouse model of diabetes-induced neuropathy, astrocytic, but not microglial, activation is seen in the spinal cord (Liao et al., 2011).

4. Microglia

Microglia are often considered the resident macrophages of the CNS due to their shared properties with the phagocytic macrophage. It is thought microglia are of myeloid origin, invading the developing CNS during embryogenesis, as they have been shown to express the myeloid-specific transcription factor PU.1 and microglia in an activated state are found to have a common expression of cell surface markers as macrophages and myeloid-derived immature dendritic cells (Chan et al., 2007, Ransohoff and Perry, 2009). Additionally microglia are absent from the CNS of mice deficient in PU.1 (McKercher et al., 1996). Other studies which have sought to address the question of microglial origin have used the technique of irradiation chimerism to examine the way in which the CNS was repopulated. However data from these studies must be considered carefully as it has since become evident that this technique disrupts the normal functioning of the blood-brain barrier, and irradiation-induced cell death may lead to fluxes of cytokines which would alter the responses of repopulating haematopoietic cells (Ransohoff and Perry, 2009).

For many years microglia were thought to exist in either a resting quiescent or activated phenotype, with the implication of an “all-or-nothing” role. Although these terms remain in regular use, it is now accepted that this is an oversimplistic view, specifically with regard to the “resting” state. Using two-photon microscopy, Nimmerjahn *et al.* and Davalos *et al.* examined the activity of microglia in the live adult mouse brain (Davalos et al., 2005, Nimmerjahn et al., 2005). The groups used a thinned skull preparation which enabled imaging up to 200 µm below the cranial surface. A transgenic mouse with the EGFP reporter gene inserted into the CX3CR1 locus enabled specific visualisation of microglia. This technique was able to show, for the first time, that far from being “resting”, microglia in the healthy brain are highly motile, apparently continually surveying their microenvironment. Baseline time-lapse imaging experiments of the healthy adult brain, spanning up to 10 hours, showed that the somata of the microglial cells remained generally fixed while their processes were observed to be highly motile. Microglial processes showed rapid extension and retraction, reaching up to several micrometres in length. It was estimated from these data that the complete brain parenchyma could be

monitored every few hours. Based on these reports, Hanisch and Kettenmann proposed a new terminology of “surveying” and “effector” microglia to better describe these dynamic microglial phenotypes (Hanisch and Kettenmann, 2007).

Under normal physiological conditions, microglia are thought to act to stabilise the CNS by surveying the local environment for stimuli and changes which may indicate a threat to the physiological homeostasis of the system such as trauma, ischaemia, and infection. The morphology of microglia under these conditions is ideally suited for this role, with a small soma and thin branching ramified processes. Whilst in this state there is low expression of surface antigens. Upon activation a number of changes take place. The ramified processes are retracted, and the small soma hypertrophies taking on an amoeboid shape. Activated microglia, in various experimental paradigms, have been shown to have the ability to secrete inflammatory molecules such as TNF α , interferons (IFN), inflammatory interleukins such as IL-1 β , nitric oxide (NO), trophic factors such as BDNF and GDNF, and reactive oxygen species (ROS). Additionally there are changes in gene expression and upregulation of cell surface proteins such as complement 3 receptor (CD3, also known as CD11b or OX42), major histocompatibility complex (MHC) class I and II, and receptors for cytokines and chemokines. These phenotypic changes are accompanied by cell proliferation, phagocytosis of cellular debris, and migration to the site of injury or threat (Honda et al., 2001, Hanisch, 2002, Takahashi et al., 2005, Garden and Moller, 2006, Hanisch and Kettenmann, 2007, Echeverry et al., 2008, Neumann et al., 2009).

4.1. *Microglia in animal models of pain*

4.1.1. *Recruitment and activation: candidate signals*

Following peripheral nerve injury, microglia have been shown to proliferate and migrate to the injured level of the spinal cord, as early as 3 days post-injury (Echeverry et al., 2008). Using bromodeoxyuridine (BrdU) as a mitotic marker Echeverry *et al.* were able to demonstrate cell proliferation in the lumbar spinal

cord, as early as 3 days after peripheral nerve injury. Immunohistochemical analysis revealed that the majority of these cells were microglia. The cells have an activated amoeboid morphology, and microgliosis can be observed both in the superficial dorsal horn and surrounding injured motor neuron of the ventral horn. Increased immunoreactivity of OX42 in these areas demonstrates a more activated phenotype of the cells also (Scholz et al., 2008). Using real-time RT-PCR methods it has been shown that microglia activation in the spinal cord in response to peripheral nerve injury occurs even earlier than this; mRNA of microglial surface markers CD11b, toll-like receptor 4 (TLR4), and cluster determinant 14 (CD14) were all shown to be upregulated as early as 4 hours after spinal nerve transection injury (Tanga et al., 2004).

It is thought that several signals may be responsible for this microglial recruitment following peripheral nerve injury or inflammation. Glutamate and SP released in the dorsal horn following activation of nociceptive neurons are able to act on NMDA and mGluR, and NK1 receptors found to be expressed on “resting” microglia (McMahon and Malcangio, 2009, Ransohoff and Perry, 2009). Additionally release of ATP, neuronal chemokines, BDNF, and other as yet unidentified mediators may be responsible for the microgliosis (McMahon and Malcangio, 2009). As previously discussed peripheral nerve injury or inflammation leads to electrical discharge at the time of the injury, and later spontaneous activity of the injured and neighbouring uninjured afferents. In several animal models of peripheral nerve injury it has been shown that nerve conduction blockade with local anaesthetic prior to the injury is able to prevent microglial activation (Wen et al., 2007, Hathway et al., 2009, Suter et al., 2009, Xie et al., 2009), although the relative contributions of A- and C-fibres to this phenomenon remain elusive and may well differ between animal models (Hathway et al., 2009, Suter et al., 2009). Precisely how this primary afferent barrage signals to recruit and activate microglia is still unclear, although there are now several candidate molecules thought to mediate this signal, including CCL2, IFN- γ , fractalkine, neuregulin 1 (NRG1), TLR4, CCL21, and ATP.

The chemokine CCL2, also known as MCP-1, is undetectable in the DRG and spinal cord of naïve rats, but is rapidly upregulated following peripheral nerve

injury (Flugel et al., 2001, Tanaka et al., 2004, Zhang and De Koninck, 2006, Thacker et al., 2009, Van Steenwinckel et al., 2011). Expression of MCP-1 was seen from 1 day post-injury in the ipsilateral DRG and from 3 days post-injury in the ipsilateral dorsal horn following chronic constriction injury, suggesting anterograde axonal transport to central nerve terminals (Zhang and De Koninck, 2006). This is supported by evidence that tight ligation of the L5 spinal nerve as well as the L5 dorsal root leads to accumulation of CCL2 immunoreactivity proximal to the spinal nerve ligature, and distal to the dorsal root ligature suggesting injury-induced transport of CCL2 anterogradely towards both the periphery and dorsal horn of the spinal cord (Thacker et al., 2009). Closer investigation revealed that CCL2 is mainly upregulated in small, non-peptidergic neurons of the DRG co-expressing marker of neuronal damage ATF-3 following peripheral nerve injury (Tanaka et al., 2004, Zhang and De Koninck, 2006, Thacker et al., 2009). Evoked release of CCL2 from *ex vivo* isolated dorsal horn slices with dorsal roots attached has been shown to be significantly increased following peripheral nerve injury (Thacker et al., 2009). Following chronic constriction injury, the upregulation of CCL2 expression has been shown to be temporally related to both the microgliosis observed following peripheral nerve injury, and the consequent mechanical hypersensitivity (Zhang and De Koninck, 2006), and mice deficient in CCL2 receptor, CCR2, fail to develop either mechanical hypersensitivity or spinal microgliosis in response to partial sciatic nerve ligation (Seltzer) injury (Abbadie et al., 2003, Zhang et al., 2007). Additionally intrathecal administration of CCL2 leads to a long-lasting mechanical, but not thermal, hypersensitivity in rats accompanied by an increase in OX-42 immunoreactivity, whereas spinal pre-treatment with a CCL2 neutralising antibody prior to CCI injury in rats attenuated the subsequent mechanical hypersensitivity and microgliosis compared to control IgG-treated rats (Thacker et al., 2009).

More recently IFN- γ has been suggested to play an important role in microglial recruitment and activation. IFN- γ has been shown to activate primary cultured microglial cells (Hanisch and Kettenmann, 2007), and IFN- γ levels have been shown to increase in the spinal cord after peripheral nerve injury (Tanga et al.,

2005). Using in situ hybridisation techniques Tsuda *et al.* showed that IFN- γ mRNA is present in the dorsal horn of the uninjured lumbar spinal cord, and this signal co-localises with microglial marker ionised calcium-binding adapter molecule-1 (IBA1). Intrathecal injection of IFN- γ in wild-type, but not IFN- γ receptor deficient, mice led to a long-lasting mechanical hypersensitivity and spinal microgliosis, an effect inhibited by minocycline pre-treatment. The response of IFN- γ receptor deficient mice to peripheral nerve injury was also investigated. Development of mechanical hypersensitivity and spinal microgliosis was significantly impaired in IFN- γ receptor deficient mice compared to wild-type mice, further suggesting a role for IFN- γ in nerve injury-induced microglial recruitment and activation. Finally both intrathecal administration of IFN- γ and peripheral nerve injury were shown to upregulate P2X4 receptor expression, a marker of microglial activation (discussed later, see section 4.1.2), in wild-type mice, but not in mice lacking the IFN- γ receptor (Tsuda *et al.*, 2009b).

Fractalkine, or CX3CL1, is a neuronal transmembrane glycoprotein with a chemokine domain expressed constitutively by neurons in the brain, spinal cord, and DRG. Its only receptor, CX3CR1 is expressed by microglia (Verge *et al.*, 2004). Fractalkine has been shown to be chemotactic in cultured microglia (Harrison *et al.*, 1998). This chemokine is active when membrane-bound, but may also act as a soluble chemokine following proteolytic cleavage by Cathepsin S, released by activated microglia (Clark *et al.*, 2007, Clark *et al.*, 2009). Intrathecal administration of fractalkine has been shown to cause mechanical hypersensitivity as well as a marked microgliosis and activation of p38 MAPK in spinal microglia, a marker of microglial activation (discussed later, see section 4.1.3). Conversely intrathecal administration of a CX3CR1-neutralising antibody prior to nerve injury attenuated the development of mechanical hypersensitivity and p38 MAPK activation in microglia (Zhuang *et al.*, 2007). Additionally CX3CR1 knockout mice have been shown to have deficits in the development of both inflammatory and neuropathic pain (Staniland *et al.*, 2010). More recently it has been suggested that injury-induced upregulation of CX3CR1 expression, and subsequent p38 MAPK activation is mediated by the action of IL-6. Administration of an IL-6-

neutralising antibody prior to CCI injury in rats, and on intermittent subsequent days, attenuated the development of both mechanical and thermal hyperalgesia as well as the injury-induced upregulation of both CX3CR1 and activated p38 MAPK in microglia. Intrathecal administration of IL-6 in naïve rats caused behavioural hypersensitivity, as well as upregulation of CX3CR1 and p38 MAPK activation in the spinal cord (Lee et al., 2010a). Following peripheral nerve injury Cathepsin S expression has been shown to be upregulated in the ipsilateral dorsal horn in a temporal profile similar to that of the observed microgliosis, significantly increasing 3 days post-injury, peaking 7 days post-injury, and beginning to decline 14 days post-injury (Clark et al., 2007). Pharmacological inhibition of Cathepsin S, via continuous intrathecal infusion, reduced injury-induced mechanical hypersensitivity only 7 and 14 days after injury when Cathepsin S expression was at its peak (Clark et al., 2007). Thus although Cathepsin S expression is seen earlier, the fractalkine-CX3CR1 signalling system may be of more importance at later time-points. A role for fractalkine and Cathepsin S in microglial activation and subsequent behavioural hypersensitivity has also been shown in collagen-induced arthritis and intrathecally administered LPS-induced hindpaw hypersensitivity (Nanki et al., 2004, Staniland et al., 2010, Clark et al., 2012).

Calvo *et al.* have recently shown that neuregulin 1 (NRG1) signalling through erbB2, 3, and 4 receptors in microglia promotes proliferation and chemotaxis. Intrathecal treatment with NRG1 in rats led to a microglial proliferation within the dorsal horn, as assessed by BrdU immunohistochemistry. Behaviourally the NRG1-treated animals developed a cold and mechanical hypersensitivity. Using *in situ* hybridisation techniques, the authors found clear expression of NRG1 in both small and large diameter DRG neurons. Only low levels were found to be expressed in dorsal horn neurons, thus it was concluded that the most likely source of NRG1 within the dorsal horn was from the primary afferent terminals of DRG cells. To determine the extent of the role of NRG1 in injury-induced gliosis of the spinal cord, erbB2 receptor inhibition and the sequestration of endogenous NRG1 were employed in rats with L5 SNT injury. Both strategies reduced injury-induced behavioural hypersensitivities and the extent of the microgliosis (Calvo et al., 2010). Subsequent work showed that

the proliferative effect of NRG1 was mediated via the MEK/ERK/1/2 pathway, and the chemotactic effect was dependent on the PI3K/Akt signalling pathway. Interestingly the p38 MAPK pathway was shown not to play a role (Calvo et al., 2011).

TLR4 is a member of the family of toll-like receptors; a family of 12 evolutionary conserved membrane proteins that are fundamental to the innate immune response (Trinchieri and Sher, 2007). It has been shown that an increase in spinal microglial TLR4 activation correlates with the onset of behavioural hypersensitivity in response to peripheral nerve injury (Tanga et al., 2004). Mice deficient in TLR4 showed reduced behavioural hypersensitivity in response to peripheral nerve injury compared with wild-type controls, as well as an attenuated spinal microgliosis. Additionally daily Intrathecal administration of TLR4 antisense oligodeoxynucleotide in rats following peripheral nerve injury resulted in an attenuated behavioural hypersensitivity and spinal microglial activation compared to those animals receiving control mismatch oligodeoxynucleotide (Tanga et al., 2005). CD14 is an accessory protein which amplifies the TLR4 response to pathogens. CD14 knockout mice also show a decreased behavioural sensitivity to peripheral nerve injury, and intrathecal injection of CD14 in mice can lead to mechanical hypersensitivity (Cao et al., 2009), further implicating TLR4 as having a role in nerve injury-induced microgliosis. TLR4 is a pattern recognition receptor (PRR), meaning it recognises and binds to a range of patterns or motifs associated with pathogens or danger such as the release of nucleic acids and proteins released after cell damage. Peripheral nerve injury may lead to the release of such substances, leading to TLR4 activation, ultimately leading downstream to the release of pro-inflammatory cytokines such as IFN- γ , IL-1 β , and TNF- α , expression of which was significantly reduced after injury in the TLR4 KO mice compared to wild-type control (Tanga et al., 2005). These cytokines may serve to further activate and recruit the microglial response.

The chemokine CCL21, or secondary lymphoid-tissue chemokine (SLC), is rapidly upregulated in damaged neurons (Biber et al., 2001), and has been shown to be upregulated in C-fibres in the injured DRG following L5 SNT in

rats (Biber et al., 2011). CCL21 in neurons is sorted in vesicles and transported to nerve terminals via the Golgi and trans-Golgi network (de Jong et al., 2005). Indeed, after L5 SNT staining for the chemokine was initially detected only in the DRG, 12 hours after injury, but was later detected in the nerve terminals of the primary afferents of the dorsal horn 1 and 2 days after nerve injury indicating axonal transport to the terminals (Biber et al., 2011). CCL21 acts via CXCR3 receptors on microglia to induce chemotaxis *in vitro* (Rappert et al., 2002, de Jong et al., 2005). It is unlikely to be responsible for microglial recruitment following nerve injury, however, and instead appears to be more important in microglial activation. Mice deficient in CCL21 did not develop mechanical hypersensitivity following L5 SNT, though were shown to have a comparable microglial response to that of wild-type mice following L5 SNT injury, with comparable morphology, levels of proliferation, and OX42 immunoreactivity. However microglial P2X4 receptor upregulation (discussed later, see section 4.1.2), seen in wild-type mice, was absent in CCL21-deficient mice. Intrathecal injection of CCL21 2 days after nerve injury in these mice did result in P2X4 receptor upregulation, however, suggesting that the downstream effects of CCL21 are mediated via the P2X4 receptor pathway (Biber et al., 2011). Interestingly although CCL21 deficient mice failed to develop mechanical hypersensitivity in response to peripheral nerve injury, development of heat sensitivity, assayed by the hot plate test, was comparable to that observed in wild-type mice, suggesting different mechanisms and pathways are responsible for the changes observed in the different modalities of behaviour.

4.1.2. Purinergic systems

A prominent signalling pathway in the development of neuropathic pain involves ATP acting on microglial purinergic receptors. ATP is known to stimulate microglial cells both *in vitro* and *in vivo* (Honda et al., 2001, Tsuda et al., 2003, Davalos et al., 2005). Using two-photon imaging techniques, Davalos *et al.* showed that focal application of ATP induced a rapid microglial response similar to that observed following laser ablation injury, a response shown to be P2Y-receptor dependent (Davalos et al., 2005). In fact microglia

express several metabotropic P2Y receptor subtypes: P2Y₁, 4, 6, and 12, as well as the P2X₄ and 7 ionotropic purinergic receptors (Trang et al., 2012). In broad terms P2Y receptors appear to regulate motility and chemotaxis in the surveillance phenotype, and phagocytosis following activation. P2X receptors, however, appear to be expressed only in activated microglia (Haynes et al., 2006, Ulmann et al., 2008).

P2X₄ receptors

A crucial role for microglial purinergic signalling in chronic pain was first demonstrated in 2003 by Tsuda *et al.* who showed a critical role for P2X₄ in initiating injury-induced hypersensitive behaviours (Tsuda et al., 2003). Following peripheral nerve injury P2X₄ was shown to be upregulated in microglia, and not neurons or astrocytes, as early as 1 day after injury, continuing for at least 14 days post-injury, a finding corroborated using eGFP labelled knock-in mice (Tsuda et al., 2003, Coull et al., 2005). Pharmacological inhibition of P2X₄ receptors 7 days after injury transiently increased mechanical paw withdrawal threshold, while intrathecal injection of cultured ATP-stimulated microglia in naïve mice led to a marked decrease in paw withdrawal threshold 5 hours later (Tsuda et al., 2003). These findings are supported by recent work showing that mice lacking the P2X₄ receptor exhibit reduced mechanical hypersensitivity subsequent to peripheral nerve injury (Ulmann et al., 2008, Tsuda et al., 2009a). As previously mentioned, several signals have been implicated in the upregulation of P2X₄ expression in animal models of nerve injury, including IFN- γ and CCL21 (Tsuda et al., 2009b, Biber et al., 2011).

P2X₄ receptor activation leads to p38MAPK-mediated BDNF release

The downstream effects of spinal microglial P2X₄ receptor stimulation have been proposed to be mediated by a release in BDNF. *In vivo* intrathecal application of ATP-stimulated cultured microglia results in behavioural hypersensitivity (Tsuda et al., 2003, Coull et al., 2005). Recordings from lamina I neurons of lumbar spinal cord slices taken from rats which had received intrathecal injection of ATP-stimulated cultured microglia revealed a depolarising shift in the anion reversal potential of these neurons, which

resulted in an inversion of polarity of GABA-induced currents (from outward to inward) (Coull et al., 2005). This hyperpolarising effect of GABA had been previously reported in lumbar spinal cord slice recordings obtained from animals which had received peripheral nerve injury, and had been attributed to a reduction in expression the potassium-chloride exporter KCC2 (Coull et al., 2003). Peripheral nerve injury-induced decrease in KCC2 expression temporally coincides with a concomitant increase in spinal BDNF expression, and BDNF-sequestration with a TrkB/Fc chimera protein prior to injury prevented both the KCC2 decrease and injury-induced behavioural hypersensitivity (Miletic and Miletic, 2008). Application of BDNF to spinal cord slices caused a depolarising shift in the anion reversal potential of lamina I neurons, and a hyperpolarising effect of subsequently applied GABA. Administration of a TrkB inhibitor prior to intrathecal application of ATP-stimulated cultured microglia in rats prevented the development of mechanical hypersensitivity. Finally by pre-treatment of the culture with siRNA directed against BDNF, which also prevented the onset of mechanical hypersensitivity, the group showed that the source of BDNF in this paradigm was from the microglial cells as opposed to neurons or other cell types within the spinal cord (Coull et al., 2005). This effect was demonstrated to be P2X4 receptor-mediated by Ulmann *et al.* Mice lacking the P2X4 receptor demonstrated an accumulation of BDNF within spinal microglia following peripheral nerve injury. In primary cultured microglia from these mice ATP stimulation did not induce BDNF release, whereas ATP stimulation of cultured microglia from wild-type mice did induce BDNF release (Ulmann et al., 2008). Experiments using primary cultured microglia show that ATP-induced BDNF release occurs in two phases, with significant release occurring in the first 5 minutes and again 60 minutes after ATP application. BDNF levels within the cells themselves showed only one phase of increase, peaking at 60 minutes post-application of ATP, suggesting the BDNF released in the first phase was from a pre-existing intracellular pool. Pre-treatment with a p38 MAPK inhibitor prevented release of BDNF in both phases following ATP application. Levels of phosphorylated p38 MAPK, the activated form of this kinase, were increased significantly 60 minutes after ATP application, but not 5 minutes post-application. The authors concluded that the initial phase of BDNF increase may be mediated by basal

p38 MAPK activity, whereas the increased production of BDNF and late phase of release was mediated by an increase in p38 MAPK activity (Trang et al., 2009).

P2X7 receptor signalling

Spinal microglial P2X7 receptor activation has also been implicated in the development of neuropathic pain-like behaviours in animal models of peripheral nerve injury. Mice lacking the P2X7 receptor show reduced behavioural hypersensitivity after partial sciatic nerve ligation (Chessell et al., 2005). Systemic pre-treatment with selective P2X7 antagonists has been shown to be effective in alleviating the behavioural hypersensitivity induced by intraplantar injection of complete Freund's adjuvant (CFA), an inflammatory model of pain (Honore et al., 2009). However P2X7 is widely expressed in several cell types (Burnstock, 2007), and this may not represent a microglial-specific mechanism of pain signalling. However microglial P2X7 receptors are likely to at least contribute to the generation of pain-like behaviours since the release of Cathepsin S has been demonstrated to be P2X7-dependent. Similar to downstream effects of P2X4 receptor signalling in microglia, this effect has been shown to be mediated by p38 MAPK activation (Clark et al., 2010b).

P2Y12 receptor signalling

P2Y12 expression levels in microglia have been shown to increase in the ipsilateral dorsal horn of peripheral nerve-injured rats (Kobayashi et al., 2008b, Tozaki-Saitoh et al., 2008). Intrathecal administration of a P2Y12 antagonist or P2Y12 antisense oligonucleotides prior to peripheral nerve injury prevented the development of behavioural hypersensitivity as well as suppressed the phosphorylation of p38 MAPK in spinal microglia, a marker of microglial activation (see section 4.1.3) (Kobayashi et al., 2008b). While P2Y12 antagonism did prevent behavioural hypersensitivity induced by peripheral nerve injury, it did not affect the associated spinal increase in OX42 immunoreactivity, indicating that P2Y12 receptors do not play a role in recruitment of microglia, rather the subsequent pathways which affect sensory processing sensitivity.

4.1.3. Microglial intracellular signalling

The MAPK family are evolutionally conserved, and play a critical role in cell signalling and gene expression. The family consists of three major members: ERK, p38, and JNK kinases. Following L5 SNT in rats, it has been shown that ERK is sequentially activated in neurons, microglia, and astrocytes. In the first few hours after injury phosphorylated ERK was seen in neurons in the ipsilateral superficial dorsal horn. Microglial expression of active ERK peaked between 1 and 3 days after surgery, but was still present 10 days after surgery. 10 days after surgery ERK expression was also observed in astrocytes. Active ERK expression was predominantly seen in astrocytes only in the spinal cord by 21 days after surgery (Zhuang et al., 2005). Intrathecal administration of a MEK inhibitor 2, 10, and 21 days after surgery attenuated injury-induced behavioural hypersensitivity (Zhuang et al., 2007). ERK activation in microglia has also been demonstrated in the streptozotocin-induced diabetes model of neuropathic pain. Inhibition of the pathway was shown to reduce behavioural hypersensitivity in this model also (Tsuda et al., 2008).

Another major signalling pathway implicated in peripheral injury-induced microgliosis is the p38 MAPK pathway, and the active form of the kinase phosphorylated p38 (hereafter referred to as p-p38) is often used as a marker of microglial activation in animal models of pain. Following peripheral nerve injury, it has been shown that p-p38 levels increase in the ipsilateral dorsal horn from 1 day post-injury, peak around 3 days, and continue to be elevated up to 3 weeks post-injury. Immunohistochemical analysis has shown that this increase occurs in microglia, and not astrocytes or neurons, at these time points (Jin et al., 2003, Tsuda et al., 2004). Pharmacological inhibition of p38 MAPK is able to prevent, but not reverse, pain-like behaviours and spinal microgliosis. Similarly pre-treatment with bupivacaine microspheres prevented, but did not reverse, dorsal horn increases in p-p38. Presumably this was due to the prevention of the microgliosis observed (Jin et al., 2003, Svensson et al., 2003, Tsuda et al., 2004, Wen et al., 2007).

Activated p38 is translocated to the nucleus, where it phosphorylates transcription factors such as ATF-2. IL- β , IL-6, TNF- α , cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and several other inducible cytokines are upregulated in response to p38 activation, many of which have been shown to be pro-nociceptive (Kumar et al., 2003, Svensson et al., 2003, Sung et al., 2005, Ji and Suter, 2007, Matsui et al., 2010). p38 activation has also been demonstrated to have post-translational effects since application of lipopolysaccharide (LPS) to *ex vivo* spinal cord slices has been shown to induce rapid release of IL-1 β via p38 activation, an effect abolished by pre-treatment with a p38 MAPK inhibitor (Clark et al., 2006). Several mediators have been shown to activate p38 MAPK in microglia, including TNF- α , IL-1 β , fractalkine, MCP-1, and iNOS (Sung et al., 2005, Clark et al., 2007, Zhuang et al., 2007, Clark et al., 2010b). Activation of purinergic receptors P2X4, P2X7, and P2Y12 has also been shown to activate p38 (Kobayashi et al., 2008b, Trang et al., 2009). It is of interest to note that several p38 activators are also upregulated by p-p38, generating a positive feedback loop.

4.1.4. Microglial mediators

Several of the mediators shown to be released by activated microglia are the same as those that are known to bring about further microglial activation, in a positive feedback loop that no doubt amplifies the microglial response to peripheral nerve damage. These include TNF- α , IL-1 β , and IL-6 (Sweitzer et al., 2001, Hanisch and Kettenmann, 2007, Lee et al., 2010a, Zhang et al., 2011). TNF- α and IL-1 β both upregulate iNOS expression in microglia and neurons (Schomberg and Olson, 2012). The resultant production of diffusible NO is thought to modulate synaptic plasticity by post-translational modifications to the NMDA receptor (Meller and Gebhart, 1993). Pharmacological inhibition of NO production has been shown to reduce thermal hypersensitivity in rats after CCI injury although this would have blocked all forms of NOS, including microglial iNOS (Meller et al., 1992). A recent study showed that TNF- α and IL-1 β applied to isolated lumbar spinal cord slices led to an enhancement of excitatory AMPA and NMDA-induced currents. Additionally IL-1 β , as well as IL-6, suppressed inhibitory GABA and

glycine-induced currents (Kawasaki et al., 2008b). Similar data have been obtained in the examination of dorsal root ganglion neurons (Ozaktay et al., 2006). In addition to these pro-inflammatory cytokines, other pro-inflammatory cytokines and algescic mediators released from activated microglia include IL-15, IL-17, IL-18, PGE₂, leukotrienes, NO, and BDNF. Evidence for a role of IL-1 β in pain signalling will be discussed in detail in a later section.

It is now well accepted that injury-induced microglial activation contributes to the initiation of pain states, evidenced by the effectiveness of pre-treatment with inhibitors such as minocycline. However it is also now emerging that there are mechanisms in place which may limit the extent of microglial activation. Anti-inflammatory cytokines such as IL-10, IL-4, and TGF- β have all been shown to have the potential to inhibit activated microglial responses (Austin and Moalem-Taylor, 2010). This is perhaps not so surprising given that the role of microglial activation should ultimately be to recruit and effect an immune response, via secretion of inflammatory cytokines and upregulation of cell surface receptors such as complement receptor C5a, which should remove the perceived threat. However once the job of the immune response is complete, there should be some mechanism to “call off” the immune response and allow the system to return to a normal homeostasis.

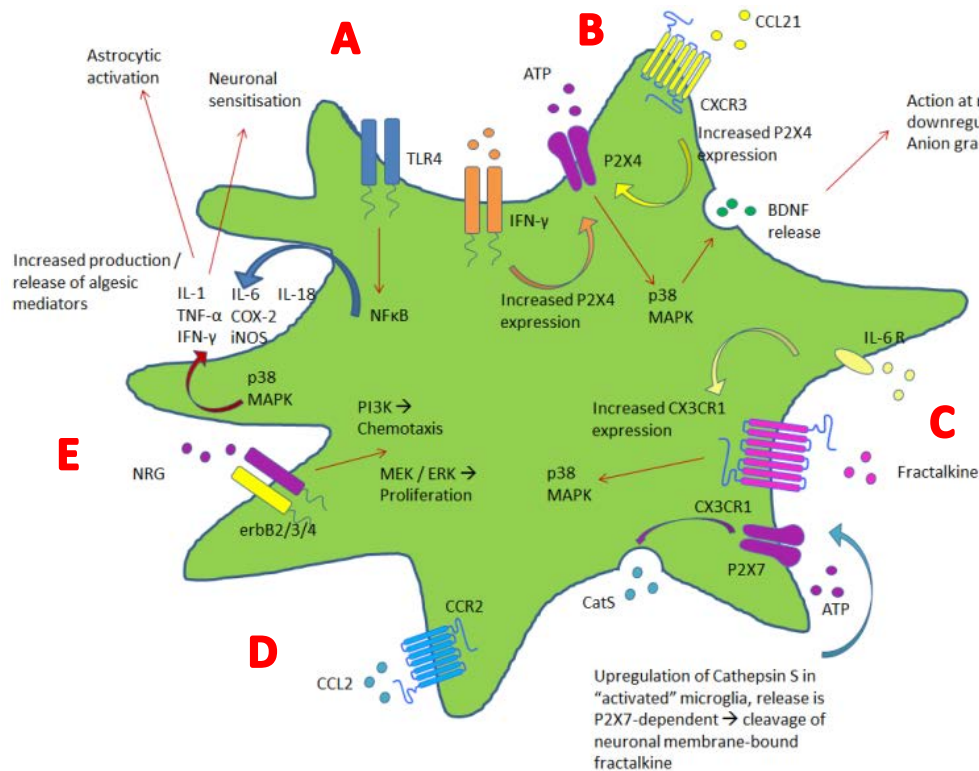


Figure i.5: Overview of the microglial activation in animal models of pain

A: TLR4 activation by unknown agonists leads to NFκB activation which leads to the upregulation and release of various known algesic mediators. p38 MAPK activation has also been shown to upregulate production of algesic mediators. These mediators are able to sensitise neurons, and activate astrocytes leading to their proliferation and secretion of inflammatory mediators. **B:** IFN-γ and also CXCR3 receptor activation lead to increased P2X4 receptor expression. P2X4 receptor activation leads to the upregulation of p38 MAPK which in turn leads to an increase in BDNF production and release. BDNF action at neuronal TrkB receptors causes a downregulation of KCC2, resulting in an anion gradient shift. **C:** Reactive microglia increase expression and release of the protease Cathepsin S which cleaves, and liberates, neuronal membrane-bound fractalkine. Release of Cathepsin S is P2X7-dependent. IL-6 receptor activation leads to increased CX3CR1 expression, the receptor at which fractalkine acts. CX3CR1 receptor activation leads to p38 MAPK activation. **D:** CCL2, upregulated in sensory neurons following peripheral nerve damage, binds to microglial receptor CCR2. **E:** NRG1 binds to erbB2/3/4 receptors leading to activation of intracellular signalling PI3K and MEK/ERK pathways, leading to chemotaxis and proliferation of microglia respectively.

5. Astrocytes

That minocycline and other treatments targeted at inhibiting microglial activation are able to prevent, but not reverse, established pain-like behaviours is indicative of their transient role in initiating a cascade of events downstream of their own activation. One such series of events appears to be the activation of astrocytes. Unlike microglial activation, astrocytic activation in animal models of pain occurs several days after the insult, and is much longer lasting (Colburn et al., 1999, Tanga et al., 2004, Romero-Sandoval et al., 2008).

Under normal physiological conditions astrocytes act to “mop up” molecules that are toxic or are in excessively high concentration, keeping the extracellular homeostasis. Removal of excess extracellular potassium during neuronal signalling is mediated by its uptake into adjacent astrocytes. This potassium is later released back into the extracellular space in a regulated fashion (Kofuji and Newman, 2004). Additionally astrocytes are thought to be able to regulate neurotransmitter release via release of calcium from internal stores (De Leo et al., 2006). Following peripheral nerve injury astrocytes enlarge, and upregulate intermediate filament protein GFAP, vimentin, and the calcium-binding peptide S100 β (Ridet et al., 1997, Pekny and Nilsson, 2005, Tanga et al., 2006). Mice deficient in GFAP do develop pain-like behaviours normally after peripheral nerve injury, although this is shorter-lasting than that observed in wild-type mice. Additionally the administration of GFAP antisense treatment in rats 6 weeks after peripheral nerve injury reversed established behavioural hypersensitivity. Both these findings point towards a role in maintenance of neuropathic pain for astrocytes (Kim et al., 2009). Like microglia, activated astrocytes secrete a number of pro-inflammatory mediators which either directly excite neurons or modulate activation thresholds, including excitatory amino acids, ATP, IL-1 β , TNF- α , IL-6, CCL2, NGF, and glutamate (Sweitzer et al., 1999, Gao et al., 2009, Austin and Moalem-Taylor, 2010).

As discussed earlier, ERK activation in astrocytes following peripheral nerve injury has been observed 10 and 21 days after surgery, and injection of a MEK (ERK kinase) inhibitor at both these time points reduced associated mechanical hypersensitivity (Zhuang et al., 2005). Additionally JNK activation

has been described in activated astrocytes following peripheral nerve injury. Expression of this MAP kinase is more restricted than that of the other two in the family, p38 and ERK. Following L5 SNT JNK was briefly upregulated in the injured DRG 12 hours after injury and was later upregulated exclusively in astrocytes in the spinal cord 3 days after injury, persisting to the end of the study (21 days after injury). Infusion with an inhibitor of JNK was able to reverse established mechanical hypersensitivity 10 days after injury. However the injury-induced upregulation of GFAP was unaffected by this treatment suggesting that JNK is not responsible for the proliferation of astrocytes following peripheral nerve injury (Zhuang et al., 2006). Basic fibroblast growth factor (bFGF) is an astrocyte activator that has been implicated in activation of the JNK pathway. After peripheral nerve injury bFGF is upregulated in injured DRG neurons (Ji et al., 1995), and intrathecal infusion of bFGF is sufficient to produce mechanical hypersensitivity in rats (Ji et al., 2006).

The precise mechanisms behind the proliferation and activation of astrocytes following peripheral nerve injury are unclear. One possible mechanism may be related to injury-induced upregulation of bFGF. Another algescic mediator which has recently gained attention as a possible mediator of astrocytic activation is IL-18. Recently Miyoshi and colleagues showed that following peripheral nerve injury there is a marked upregulation of both IL-18 and IL-18 receptor in the spinal cord, and in microglia and astrocytes respectively. Administration of an IL-18 and IL-18 receptor neutralising antibody reduced nerve injury-induced mechanical hypersensitivity, as well as astrocytic activation as assessed by NFkB activation and GFAP immunoreactivity. Conversely, intrathecal injection of IL-18 in naive rats caused mechanical hypersensitivity and upregulation of both GFAP and IBA1 in the dorsal horn of the spinal cord. Thus it was concluded that IL-18 released from activated microglia in the spinal cord following peripheral nerve injury subsequently activates astrocytes through an NFkB-dependent signalling pathway (Miyoshi et al., 2008). Interestingly IL-18 was initially known as IFN- γ -inducing factor, owing to its ability to cause release of the cytokine from immune cells, and indeed has been shown to cause upregulation of IFN- γ mRNA and protein in cultured microglia and astrocytes (Kawanokuchi et al., 2006). This may be another mechanism by

which the response of activated microglia, following peripheral nerve injury, may be amplified as IFN- γ receptor activation in microglia has been shown to play an important role in peripheral nerve injury-induced microgliosis (Tsuda et al., 2009b).

6. *Endogenous anti-inflammatory mediators*

As previously mentioned, as well as a pro-inflammatory phase, an inflammatory response should also consist of a resolution phase. It is now accepted that this is an active phase of the inflammatory response, mediated by pro-resolution molecules (Serhan and Savill, 2005, Serhan et al., 2008). Lipoxins, derived from arachidonic acid, and resolvins and protectin, derived from omega-3 essential polyunsaturated fatty acids, have recently been identified as pro-resolution molecules. Anti-inflammatory actions of these molecules include the promotion of phagocytosis of dead cells by macrophages, the cessation of production of chemoattractants, and the departure of inflammatory cells from the site of inflammation via the lymphatics (Levy et al., 2001). The role of pro-inflammatory molecules in pain has begun to generate interest. Intrathecal pre-treatment with Resolvin E1 has been shown to attenuate the development of peripheral nerve injury-induced hypersensitivity by preventing the microglial inflammatory response (Xu et al., 2012). Additionally intrathecal application of LipoxinA₄ prevented development of behavioural hypersensitivity following chronic compression of DRG, and prevented the spinal upregulation of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6. Thus a pharmacological approach aimed at enhancing the pro-resolution phase of inflammation may prove beneficial in the treatment of neuropathic pain (Sun et al., 2012a).

7. *Interleukin-1 β* and *Interleukin-18*

7.1. *Interleukin-1 β*

IL-1 was first described in the 1940s as the pyrogenic mediator released from the leukocytes of rabbits (Dinarello, 2010), and is the founding member of the IL-1 family of cytokines, which at present consists of 11 members including IL-1 α , IL-1 β , IL-1Ra, IL-18, and IL-33 (Dinarello, 2009). A primary function of the IL-1 family is to elicit, but also control, a pro-inflammatory reaction in response to danger signals in the form of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) as part of the innate immune response. Molecules and molecular patterns which are expressed by microbes, but which are not normally associated with mammalian cells, are called PAMPs. Examples of PAMPs include LPS, a component of cell wall of gram negative bacteria, peptidoglycan, a component of cell wall of gram positive bacteria, bacterial flagellin, and unmethylated CpG DNA of bacteria and viruses. DAMPs are typically molecules released from damaged or dying cells such as uric acid crystals or ATP which indicate danger, possibly related to infection or tissue injury (Martinon et al., 2009).

The inflammatory mediator first described as IL-1 was subsequently determined to be two proteins; IL-1 α and IL-1 β . Although the products of two different genes, IL-1 α and IL-1 β have high sequence homology. Both are initially synthesized as larger pre-cursor proteins, which are cleaved to generate the smaller mature proteins. However pro-IL-1 α is biologically active, whereas pro-IL-1 β does not have biological activity and must be cleaved to exert its effects. Both cytokines bind to the same receptor, IL-1R1, and thus exert similar biological effects. Pro-IL-1 α is expressed constitutively in all epithelial cells, and can be found expressed on the surface of several cell types including monocytes and B lymphocytes. Like pro-IL-1 β , pro-IL-1 α lacks a leader sequence, meaning it cannot be secreted by classical ER / golgi secretion pathways. However, unlike IL-1 β , the vast majority of IL-1 α , in both its larger pro-form and cleaved mature form, remains either in the cell or membrane-bound rather than secreted to exert its effects extracellularly. It is

thought that in addition to its ability to exert inflammatory effects, pro-IL-1 α acts as an autocrine growth factor in fibroblasts and endothelial cells (Dinarello, 2009). In fact both IL-1 α and IL-1 β are capable of rapidly inducing the mRNA expression of hundreds of genes, including the expression of their own genes (Jura et al., 2008, Weber et al., 2010).

In addition to IL-1 α and IL-1 β there is a third IL-1 protein, IL-1 receptor antagonist (IL-1Ra). This molecule is structurally related to both IL-1 α and IL-1 β , and binds the same receptors with similar specificity and affinity. However it lacks biological activity owing to its inability to interact with IL-1RAcP (interleukin-1 receptor accessory protein), a necessary step in IL-1 signalling. Thus IL-1Ra represents the first of several controls within the IL-1 family limiting the IL-1 inflammatory response. (Arend et al., 2008, Weber et al., 2010). In addition to IL-1R1, the IL-1 ligands may also bind IL-1R2. Structurally similar to IL-1R1, IL-1R2 lacks a signalling-competent cytosolic domain and thus acts as a decoy receptor. The inflammatory effects of extracellularly released IL-1 ligands can be further controlled by the shedding of the extracellular IL-1-binding domains of the receptors leading to soluble IL-1R1 (sIL-1R1), soluble IL-1R2 (sIL-1R2), and soluble IL-1RAcP (sIL-RAcP) (Subramaniam et al., 2004, Dinarello, 2005, Weber et al., 2010).

Following binding of IL-1 α or IL-1 β to functional IL-1R1, the intracellular signalling pathway is long and complex with multiple levels for regulation of signalling, allowing further opportunity to regulate the effects of IL-1. A simplified schematic of IL-1 signalling is shown in Figure i.6, and described below. Functional ligand binding to IL-1R1 results in a conformational change which allows recruitment and binding of IL-1R1AcP. Following this step adaptor molecule myeloid differentiation primary response gene 88 (MyD88) is recruited to the complex via homotypic protein-protein interactions at the toll/IL—1R (TIR) domain, characteristic of proteins of the TLR superfamily. Subsequently MyD88 recruits serine/threonine kinases IL-1R-associated kinase (IRAK)-1, IRAK-2, and IRAK-4 to the complex via interactions of death domains in the kinases with a death domain at the N-terminal portion of MyD88. IRAK-4 phosphorylates IRAK-1 and IRAK-2, leading to the recruitment

of tumour necrosis factor-associated factor (TRAF)-6. The IRAK-1, IRAK-2, and TRAF-6 complex dissociates from the initial receptor complex and forms a new complex with transforming growth factor β -activated kinase-1 (TAK-1), TAK-1-binding protein (TAB)-1, and TAB-2 at the plasma membrane. IRAK-1 phosphorylates TAK-1 and TAB-1 before ubiquitination marks it for degradation at the plasma membrane causing it to dissociate from the complex. The remaining complex translocates to the cytosol where ubiquitination of TRAF-6 leads to TRAF-6-induced activation of TAK-1. TAK-1 is then able to phosphorylate cytosolic MAP kinases, as well as the IKK complex (inhibitor of NF κ B kinase). The IKK complex consists of IKK- α , IKK- β , and IKK- γ (also sometimes referred to as NF κ B essential modulator (NEMO)). The activated IKK complex phosphorylates I κ B, which leads to its ubiquitination and subsequent degradation. I κ B associates with, and inhibits, NF κ B. Its degradation allows NF κ B to translocate to the nucleus and induce expression of target genes. NF κ B binds to a conserved DNA motif found in numerous IL-1-responsive genes, including IL-1, IL-6, IL-8, MCP-1, and COX-2. Activation of TAK-1 can also lead to the activation of JNK and p38 MAPK (Korherr et al., 1997, Holtmann et al., 2001, Akira and Takeda, 2004, Subramaniam et al., 2004, Sung et al., 2005, Loiarro et al., 2010, Weber et al., 2010).

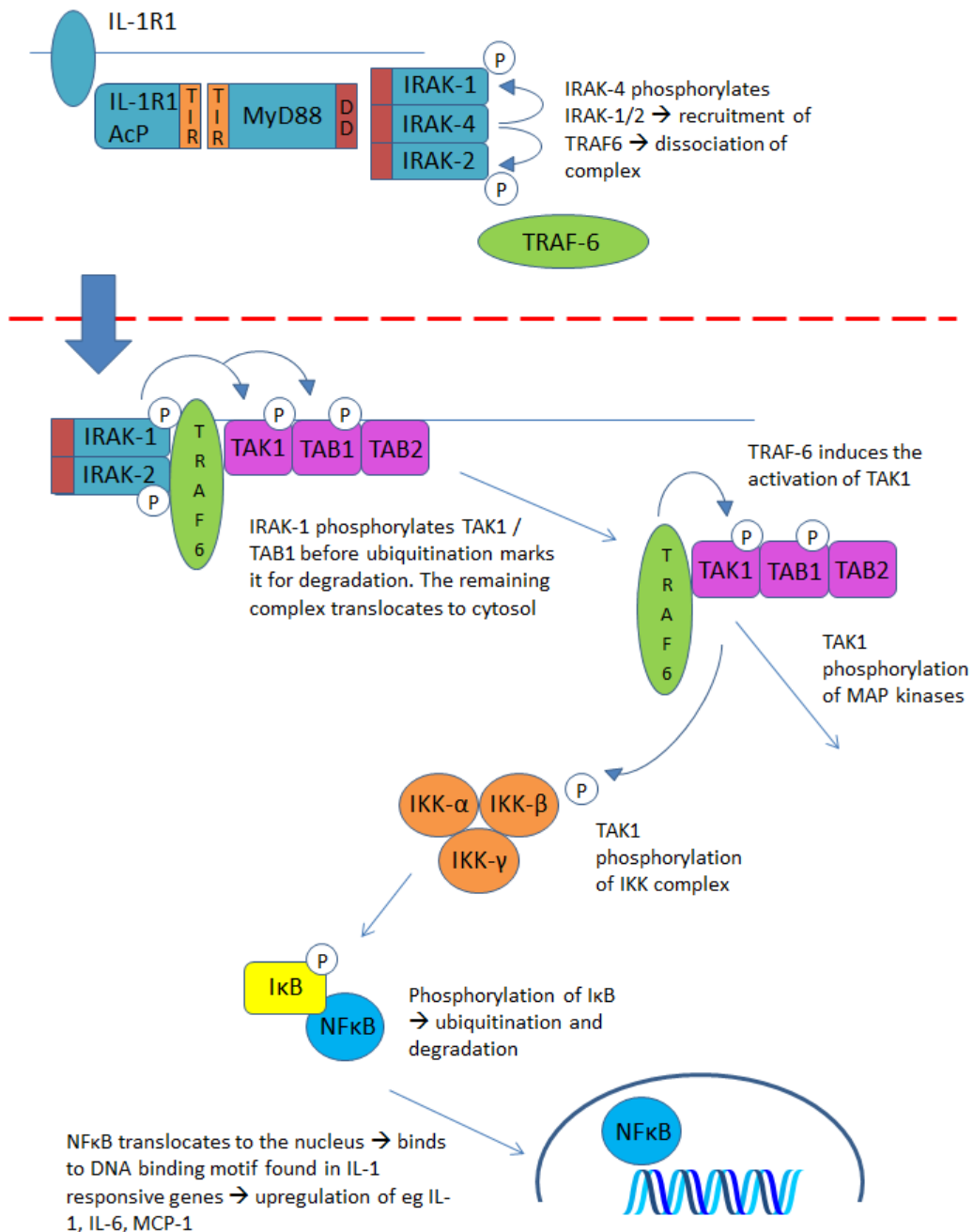


Figure i.6: Overview of IL-1R activation

Activation of IL-1R1 leads to conformational change allowing recruitment of IL-1RAcP. MyD88 is recruited to the complex via TIR-TIR interaction. Subsequently IRAK-1/2/4 are recruited by Death Domain (DD) interaction. Phosphorylation of IRAK-1/2 by IRAK-4 leads to recruitment of TRAF-6 and subsequent dissociation of the complex, and association with the TAK1/TAB1/TAB2 complex. IRAK-1 phosphorylates TAK1 and TAB1. The TRAF-6, TAK1/TAB1/TAB2 complex translocates to the cytosol where TRAF-6 activates TAK1. TAK1 phosphorylates cytosolic MAP kinases as well as the IKK complex. Phosphorylation and activation of the IKK complex leads to phosphorylation of the inhibitory IκB. IκB is degraded allowing NFκB to translocate to the nucleus.

7.1.1. Interleukin-1 β and pain

IL-1 β has been shown experimentally to have the ability to cause pain-like behaviours in rodents at multiple levels of the pain pathway. Following peripheral nerve injury, expression of both IL-1 α and IL-1 β are quickly upregulated at the site of injury (Okamoto et al., 2001). Intraplantar injection of IL-1 β has been showed to elicit rapid behavioural hypersensitivity, an effect accompanied by a decrease in activation thresholds of DRG neurons to touch stimuli and a significant increase in firing response to touch, cold, and heat stimulations of the periphery (Fukuoka et al., 1994, Cunha et al., 2000). Additionally, intraplantar injection of inflammatory stimuli such as LPS and complete Freund's adjuvant (CFA) leads to an upregulation of inflammatory cytokines, including IL-1 β , at the site of injection as well as in the DRG and spinal cord (Cunha et al., 2000, Samad et al., 2001). Application of IL-1 β in the spinal cord and brain have been shown to cause thermal hypersensitivity, and potentiation of responses of WDR neurons (Oka et al., 1993, 1994, Sung et al., 2004, Kawasaki et al., 2008b). IL-1 β expression has been shown to be increased at several levels of the pain pathway in several animal models of neuropathic pain (Milligan et al., 2001, Lee et al., 2004, Clark et al., 2006, Uceyler and Sommer, 2008). Inhibition of IL-1 β signalling by IL-1RA or neutralising antibodies against the IL-1 β or IL-1R have been shown to attenuate pain-like behaviours (Sommer et al., 1999, Sweitzer et al., 2001, Zhang et al., 2008a). IL-1 β is thought to exert its hyperalgesic effects both indirectly, via its ability to induce expression of a variety of proteins associated with pain signalling including PGE₂, COX2, NO, IL-6, and MCP-1 (Jura et al., 2008, Weber et al., 2010), and also directly by enhancing calcium and non-selective cation currents, and inhibiting potassium efflux (Kawasaki et al., 2008b, Zhang et al., 2008a, Austin and Moalem-Taylor, 2010). In the spinal cord, application of IL-1 β leads to an increase in excitatory AMPA and NMDA-induced currents, through PKC mediated phosphorylation of NR1 and NR2B subunits, and decreases spontaneous GABA and glycine inhibitory currents (Kawasaki et al., 2008b, Zhang et al., 2008a).

7.2. Interleukin-18

IL-18, a member of the IL-1 family, is structurally related to IL-1 β and was originally identified as an IFN- γ -inducing factor. Similar to IL-1 β , IL-18 is synthesised as a 24kDa pre-cursor lacking a leader sequence, pro-IL-18, which must be enzymatically cleaved by caspase-1 to generate the mature 18kDa active cytokine. However, unlike pro-IL-1 β , pro-IL-18 mRNA is constitutively expressed (Gracie et al., 2003, Dinarello, 2009). Alone, IL-18 is able to induce low levels of IFN- γ , though this effect is greatly enhanced in the presence of IL-12 (Munder et al., 1998). Caspase-1-deficient mice injected with endotoxin have significantly lower levels of circulating IFN- γ compared to wild-type littermates (Fantuzzi et al., 1999). As well as a role in IFN- γ induction, IL-18 has a variety of other effects on the immune system, including a role in T and NK cell maturation and cytokine production (Gracie et al., 2003). Additionally IL-18 is thought to play a role in homeostasis, and dysfunction of IL-18 signalling has been implicated in a variety of diseases including metabolic syndromes, inflammatory arthritis, and multiple sclerosis (Gracie et al., 2003, Arend et al., 2008).

IL-18 signals via the IL-18R complex in a similar manner to IL-1, and TLR, signalling. Briefly IL-18 binds to IL-18R α , which then binds to IL-18R β . The complex then recruits and binds MyD88, which recruits the IRAK kinases in a similar fashion to IL-1R, and TLR, signalling. MyD88 binds IRAK1-4, which in turn recruit TRAF6. IRAK phosphorylation of the IKK complex leads to the degradation of I κ B. The dissociation of I κ B from NF κ B allows NF κ B to translocate to the nucleus and induce expression of target genes. TRAF-6 activation is also able to induce p38 MAPK activation (Gracie et al., 2003). Also analogous to IL-1, there are endogenous mechanisms to restrict IL-18 signalling. Like pro-IL-18, IL-18-binding protein (IL-18BP) is constitutively expressed. IL-18BP inhibits IL-18-induced IFN- γ production *in vitro*, and LPS-induced IFN- γ production *in vivo* (Aizawa et al., 1999, Faggioni et al., 2001). IL-18BP production is inducible by IFN- γ , negatively regulating the effects of active IL-18 (Paulukat et al., 2001).

7.2.1. Interleukin-18 and pain

As previously discussed (see section 5), IL-18 has been implicated in playing a role in mediating pain. Intrathecal administration of IL-18 in rats leads to behavioural hypersensitivity, while inhibition of IL-18 in the spinal cord suppresses nerve injury-induced mechanical hypersensitivity (Miyoshi et al., 2008). Additionally IL-18 is increased in the synovium of patients with rheumatoid arthritis (Arend et al., 2008).

8. The Inflammasome – A molecular platform for the activation of inflammatory caspases

As previously mentioned, IL-1 β is synthesised as a 33kDa inactive precursor, pro-IL-1 β , that must be enzymatically cleaved to generate mature active 17kDa IL-1 β . Although it had been known for several years that caspase-1 (initially called interleukin-1 converting enzyme (ICE)) is necessary for IL-1 β maturation in a variety of experimental paradigms, it is only more recently that the mechanisms behind this association are being elucidated. In 2002, Martinon *et al.* showed that a novel protein, Nod-like receptor protein-1 (NLRP1), along with an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), provide a platform for the activation of caspase-1. Analogous to a similar protein complex involved in the activation of caspase-9 and apoptosis, the apoptosome, this NLRP1-containing caspase-1 activating complex was termed the inflammasome (Martinon et al., 2002). Since then the inflammasome has been extensively researched, and several types are now recognised; NLRP1, NLRP3, NLRC4 (NLR family CARD domain-containing protein 4, previously known as IPAF), and AIM2, all named for the central scaffold protein involved (Schroder and Tschopp, 2010).

The inflammasome is a multi-protein complex which acts as a regulating platform for the eventual cleavage and secretion of mature IL-1 β via activation of the enzyme caspase-1. The inflammasome consists of a central scaffold protein, for which it is named, an adaptor protein (ASC, cardinal), and caspase-1. The inflammasomes are best studied in the context of the innate

immune system. It is important for an organism to be able to discriminate self from non-self invading microorganisms. Pattern recognition receptors (PRRs) are able to recognise a range of conserved microbial motifs and are expressed in cells of the immune system such as macrophages, monocytes, and dendritic cells. They may be membrane-associated, as in the case of toll-like receptors (TLRs), or cytosolic as in the case of Nod-like receptors (NLRs). The NLRs are cytosolic sensors, recognising a wide range of both pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (Martinon and Tschopp, 2005). The NLR family of proteins is characterised by a central nucleotide-binding and oligomerisation (NACHT) domain, a region of leucine-rich repeats (LRR) in the C-terminus region, and an N-terminal caspase recruitment (CARD) or pyrin (PYD) domain. Earlier nomenclature convention named NLRP1 and NLRP3 as NALP1 and NALP3, being NACHT, LRR, and PYD containing proteins. NLRP1, NLRP3, and NLRC4 are all members of the NLR family, AIM2 is a HIN-200 family member (Schroder and Tschopp, 2010).

The central scaffold proteins of the inflammasome are able to recognise a large number of both exogenous and endogenous activators, including muramyl dipeptide (NLRP1, NLRP3), sendai virus, influenza virus, adenovirus, nigericin, silica, asbestos, ATP, monosodium urate crystals, β -amyloid (NLRP3), flagellin (NLRC4), and dsDNA (AIM2) (Bryant and Fitzgerald, 2009, Martinon et al., 2009, Latz, 2010). It is thought that agonist binding occurs at the LRR, as is the case for TLR3 and TLR5 (Martinon and Tschopp, 2005, Petrilli et al., 2005), though this has yet to be conclusively demonstrated. dsDNA binds to the C-terminal HIN domain in the case of the AIM2 inflammasome. Upon agonist activation the central scaffold protein oligomerises at the NACHT domain (Duncan et al., 2007). In the case of NLRP3 and human NLRP1 (containing a PYRIN domain; this domain is absent in mouse), adaptor proteins ASC, and possibly also cardinal, are recruited to the complex via homotypic PYRIN-PYRIN domain protein interactions. In turn, inactive caspase-1 is recruited to the complex via CARD-CARD domain interactions with the adaptor proteins. NLRP1 additionally has an N-terminus extension with a CARD domain allowing for direct interaction with caspase-1

as well as another inflammatory caspase, caspase-5 (Martinon et al., 2002). NLRC4 also contains a CARD domain and appears to be able to interact directly with caspase-1. However ASC is still required for maximal NLRC4 activation, though the exact mechanisms involved are unclear since NLRC4 lacks a PYRIN domain. A diagram demonstrating structural similarities of inflammasome scaffold proteins is shown in Figure i.7.

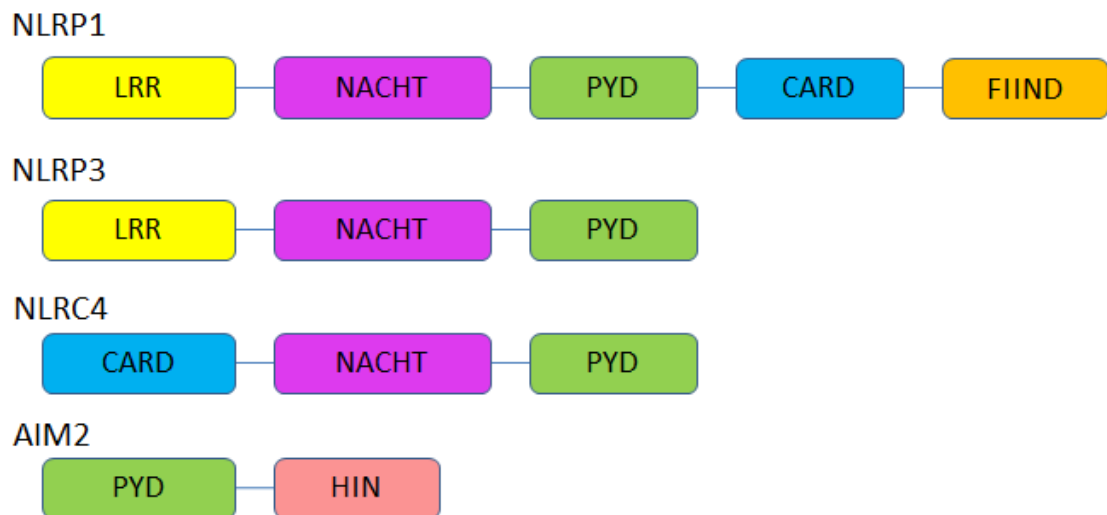


Figure i.7: Inflammasome scaffold proteins

Schematic representation of the protein domains of the inflammasome scaffold proteins NLRP1, NLRP3, NLRC4, and AIM2. Where LRR = Leucine Rich Region, NACHT = the central nucleotide binding and oligomerisation domain, PYD = Pyrin domain, FIIND = domain with function to find, HIN = HIN200 domain.

8.1. Caspase-1

Caspases are a family of cysteine proteases which cleaves proteins following an aspartate residue, the founding member of which is caspase-1. Caspase-1, then known as ICE, was originally described following attempts to purify the enzyme responsible for the cleavage of pro-IL-1 β (Thornberry et al., 1992). All caspases are synthesised as inactive pre-cursors, consisting of an N-terminal pro-domain of variable length, followed by two subunits which form the protease domain. Caspases with a large pro-domain contain homotypic protein-protein interaction motifs which function to recruit them to large multiprotein complexes; caspase-1 contains a caspase activation and recruitment domain (CARD) through which it is recruited to the inflammasome (Lamkanfi and Dixit, 2009). Pro-caspase-1 is an autolytic enzyme meaning that it is able to enzymatically cleave, and subsequently activate, other pro-caspase-1 molecules. In order for this process of activation to occur, pro-caspase-1 molecules must be recruited to the inflammasome complex where the induced proximity and dimerisation of the zymogen molecules allows this cleavage to take place. Cleavage of the pro-caspase-1 molecules results in dissociation of active caspase-1, a heterodimer complex of two p10 and two p20 subunits. Active caspase-1 is then able to cleave any pro-IL-1 β and pro-IL-18 present in the cytosol, resulting in their activation (Boatright et al., 2003, Boatright and Salvesen, 2003, Martinon and Tschopp, 2004).

As with many other steps in the interleukin-1 family inflammatory pathway, the actions of caspase-1 are tightly regulated, preventing excessive and uncontrolled release of the pro-inflammatory cytokines. In humans three endogenous inhibitors of the enzyme have been described; ICEBERG, pseudo-ICE, and INCA. All are short proteins, comprising of essentially just a CARD domain, and are encoded by caspase-like genes that have acquired a premature nonsense mutation. Thus they are able to sequester pro-caspase-1 by binding at the CARD domain, preventing its recruitment to the inflammasome complex. ICEBERG, pseudo-ICE, and INCA, are all found in the same locus as caspase-1 in humans, though are absent from the murine genome (Druilhe et al., 2001, Lamkanfi et al., 2004). Caspase-12 is thought to be a negative regulator of caspase-1 activation also. Caspase-12 is a CARD

domain-containing caspase which appears to lack any enzymatic activity. It has been shown to interact with caspase-1 and inhibit its activity. Moreover administration of various TLR ligands to splenocytes from mice deficient in caspase-12 resulted in an increased production of IL-1 β and IL-18 compared to that observed in cells from wild-type litter mates (Saleh et al., 2006). Like ICEBERG, pseudo-ICE, and INCA, caspase-12 can also be mapped to the same chromosome as caspase-1 in humans, although is also to be found in the murine genome. Following activation of caspase-1, and subsequent cleavage of pro-IL-1 β , mature IL-1 β is rapidly ejected from the cell, via non-classical secretion pathways (Brough et al., 2003, Andrei et al., 2004, Keller et al., 2008, Lopez-Castejon and Brough, 2011). The secretion of mature IL-1 β is accompanied by the secretion of active caspase-1, seen in several experimental paradigms both *in vitro* and *ex vivo* (Sanz and Di Virgilio, 2000, Clark et al., 2006, Pelegrin and Surprenant, 2006, Qu et al., 2007, May, 2008, Pelegrin et al., 2008, Qu et al., 2009). This likely reflects a further regulatory step of the inflammatory pathway, the ejection of the active caspase-1 enzyme from the cell limiting the amount of pro-IL-1 β cleavage that can take place.

8.2. ASC

Apoptosis-associated speck-like protein containing a CARD (ASC) was initially identified as a molecule which formed speck-like aggregates in HL-60 cells when apoptosis was induced by retinoic acid and other anti-tumour drugs (Masumoto et al., 1999). The protein was subsequently shown to be a short one, 195 amino acids in length, encoded by three exons; exon 1 encodes a PYRIN domain, exon 3 encodes a CARD domain, while exon 2 encodes a proline and glycine-rich domain (Matsushita et al., 2009). It is thought that this proline and glycine-rich domain imparts a bending, hinge-like, property to ASC, aiding its aggregation into the characteristic specks. A splice variant of the ASC protein, lacking the proline and glycine-rich domain, has been shown to differently regulate IL-1 β release, despite no discernible differences in caspase-1 activation, with a higher level of IL-1 β excreted from transfected Cos7 cells. Additionally ASC aggregates formed differently, with the splice variant-transfected cells exhibiting branched aggregates, compared to full-

length ASC-transfected cells which exhibited more circular aggregates (Matsushita et al., 2009).

The PYRIN domain of ASC was first shown to associate as part of a larger complex, the inflammasome, in 2002 where it was shown to be essential for pro-caspase-1 activation (Martinon et al., 2002). In support of this, several studies have shown ASC to be essential for pro-caspase-1 activation (Srinivasula et al., 2002, Mariathasan et al., 2004, Sutterwala et al., 2006, Yu et al., 2006, Yamamoto et al., 2008, Bryan et al., 2009). Unlike NLRP3, which appears to have a cytosolic localisation (O'Connor et al., 2003, Kummer et al., 2007), ASC has been shown to have a nuclear localisation in unstimulated cells (Bryan et al., 2009). Following stimulation, ASC rapidly redistributes from the nucleus to cytosol, while longer stimulation causes aggregation of ASC into speck-like structures which increase in size over time. Sequestration of ASC in the nucleus via fusion of proteins with nuclear localisation sequences greatly reduces the processing of IL-1 β following cell stimulation, demonstrating the importance of redistribution of ASC in the activation of pro-caspase-1 (Bryan et al., 2009).

In humans a small protein found at the same gene locus as ASC, POP1, with a 64% sequence homology to the PYRIN domain of ASC, acts to sequester ASC and prevent its binding to the inflammasome. POP2 has less sequence homology to ASC, but is able to bind weakly with ASC. Instead it is able to bind to several NLRPs, particularly NLRP2 and NLRP7. Again, binding of POP2 will prevent recruitment of ASC to the complex. POP1 and POP2 do not appear to be present in the murine genome (Stehlik and Dorfleutner, 2007). However a third protein, PYNOD (also known as NLRP10) is found in both the human and murine genome. PYNOD is a member of the NLR family, but lacks the LRR region. PYNOD has been shown to interact with and inhibit both ASC and caspase-1 (Wang et al., 2004a).

Although the role of ASC as an inflammasome adaptor protein is well characterised emerging evidence suggests that ASC serves a variety of roles, several of which appear to be independent of the inflammasome. ASC was

initially characterised in a search for proteins associated with the process of apoptosis (Masumoto et al., 1999). Pyroptosis is a newly-described form of programmed cell death, which is distinct from that of apoptosis. Importantly pyroptosis involves the release of inflammatory mediators, a process which is inhibited in apoptosis (Fink and Cookson, 2005). Pyroptosis was initially described in response to the intracellular infection of macrophages with *S. typhimurium*, but has subsequently been shown to occur in response to other bacterial and viral pathogens, as well as non-infectious stimulators such as LPS, MSU, R837, and nigericin (Perregaux and Gabel, 1994, Aliprantis et al., 2000, Le Feuvre et al., 2002, Fink and Cookson, 2006, Fernandes-Alnemri et al., 2007). During pyroptosis small pores form in the cell membrane leading to swelling and osmotic lysis of the cell. Prior to lysis and release of lactate dehydrogenase, a marker of cell death, processed IL-1 β is released (Brough and Rothwell, 2007). The process of pyroptosis has been shown to be dependent on caspase-1 (Fink and Cookson, 2006, Brough and Rothwell, 2007). At least in the case of ATP-stimulated pyroptosis, P2X7 receptors have been shown to be activated (Brough and Rothwell, 2007). In this paradigm, at least, the recruitment of pannexin channels by P2X7 may be responsible for the osmotic lysis occurring in pyroptosis. Similar to inflammasome formation, pyroptososome formation forms downstream of a reduction in intracellular potassium, as demonstrated by increasing extracellular potassium concentrations, or blocking potassium channels (Perregaux and Gabel, 1994, Fernandes-Alnemri et al., 2007). It is not clear what role, if any, NLRP3, or other inflammasome scaffold proteins, plays in the process of pyroptosis. It has been postulated that NLRP3 may function as an initial catalyst or enhancer of ASC oligomerisation (Fernandes-Alnemri et al., 2007). It is possible that sustained activation of inflammasome pathways eventually leads to pyroptosis, as IL-1 β is secreted from the cell prior to cell death (Brough and Rothwell, 2007). ASC specks have been observed to grow in size over time, in the absence of cell death, following primary macrophage cell stimulation which supports this idea (Bryan et al., 2009).

In addition to its role in inflammasome and pyroptososome formation, ASC has been proposed to act independently of other inflammasome components in the

regulation of MAPK phosphorylation in response to pathogens and Toll-like receptor agonists via a suppressive action of DUSP10, a dual-specificity phosphatase. DUSP10 negatively regulates the MAPK superfamily via dephosphorylation of serine/threonine residues. In macrophages derived from ASC-deficient, but not NLRP3-, NLRC4-, or caspase-1-deficient, animals MAPK activation and chemokine induction was reduced, whereas DUSP10 expression was increased in these cells. In wild-type cells, the application of DUSP10 abrogated MAPK activation and chemokine induction (Taxman et al., 2011). Finally, ASC has been shown to have a role in T cell priming in the development of arthritis in the rodent model of collagen-induced arthritis, which was also shown to be independent of other inflammasome components (Kolly et al., 2009, Ippagunta et al., 2010). Mice deficient in ASC failed to develop arthritis in this model, whereas mice deficient in both caspase-1 and NLRP3 developed arthritis normally. Collagen-specific antibodies were not detected in ASC-deficient mice as a result of a reduced antigen-specific activation of T lymphocytes by dendritic cells (Ippagunta et al., 2010). In these cases ASC is acting independently of the NLRP3 inflammasome. However it is not clear whether the protein is acting alone, or as an adaptor molecule for some other, as yet unidentified, PYRIN- or CARD domain-containing protein in these instances.

8.3. *Inflammasome expression*

Of the inflammasomes, the NLRP3 inflammasome is currently the best characterised. Despite appearing to have the largest range of activators, NLRP3 appears to have the most limited pattern of expression. NLRP3 mRNA has been demonstrated to be predominantly expressed in lymphoid organs, and organs highly populated by immune cells, such as the liver (Anderson et al., 2004, Guarda et al., 2011). NLRP3 mRNA was found in neutrophils, peripheral blood mononuclear cells, monocytes, and mast cells (Anderson et al., 2004). This is confirmed at the protein level where expression of NLRP3 has been shown in neutrophils, monocytes, and dendritic cells (Kummer et al., 2007, Guarda et al., 2011). In terms of tissue distribution NLRP3 was mainly found in non-keratinising epithelia in the oropharynx, oesophagus, and

ectocervix, as well as in the urothelial layer of the bladder. This is in contrast to the far more widespread expression of NLRP1, found in glandular epithelia structures including the gut, lung, and testis. NLRP1 was also found to be expressed in granulocytes, monocytes, dendritic cells, B and T cells, as well as neurons (Kummer et al., 2007). Owing to this more restricted cell and tissue expression and the range of endogenous danger signal activators, including ATP, it is of interest to investigate the potential contribution of the NLRP3 inflammasome in microgliosis and neuropathic pain.

It is known that in many cell types expression of NLRP3 is inducible following inflammatory stimuli such as TLR agonists and TNF- α . This is thought to reflect a further control on interleukin inflammatory signalling, preventing uncontrolled release of inflammatory cytokines. However less is known about the constitutive expression of this protein. Recently the development of a NLRP3-deficient mouse with an in-frame insertion of the enhanced GFP gene at the *Nlrp3* locus has provided greater insight into this question. In these mice the transcription of enhanced GFP is controlled by endogenous *Nlrp3* regulatory elements, allowing the expression of the fluorescent protein to act as a reporter. This study largely confirmed previous findings, showing that NLRP3 is expressed mainly in myeloid cells, including dendritic cells and monocytes. Of particular interest was the finding that different cell types appear to express differing levels of NLRP3 under resting conditions such that conventional dendritic cells and monocytes showed the highest levels of expression, with neutrophils and macrophages showing lower constitutive levels of expression. Administration of pro-inflammatory stimuli *in vivo* lead to a marked increase in GFP expression in monocytes, neutrophils, and macrophages. However there was no change observed in GFP expression in conventional dendritic cells suggesting that these cells do not further upregulate NLRP3 expression in an inflammatory environment (Guarda et al., 2011).

The secretion of pro-inflammatory cytokines is a two-step process; cells must first be primed as the expression of both IL-1 β and NLRP3 are inducible; treatment with the protein synthesis inhibitor cycloheximide dose-dependently

reduces caspase-1 activation of LPS-primed macrophages following ATP stimulation, and LPS stimulation has been shown to lead to dose-dependent increases in *Nlrp3* mRNA levels (O'Connor et al., 2003, Kahlenberg et al., 2005, Bauernfeind et al., 2009). Induction of NLRP3 expression, like pro-IL-1 β expression, can be brought about by the action of a number of different stimulators whose signalling converges at the TLR/IL-1R signalling pathways such as LPS, TLR2, and TLR7 activators (Bauernfeind et al., 2009). It has been shown that macrophages from mice deficient in MyD88 or TRIF, two alternative adaptors in the TLR4 signalling pathway, were still able to respond normally to LPS followed by ATP stimulation. However macrophages from animals deficient in both proteins failed to respond, showing a level of compensation in the TLR4-signalling pathway. The group also showed that TNF- α was able to prime cells from wild-type animals, but not from mice doubly deficient in TNFR1 and TNFR2. TNFR signalling converges on the TLR signalling pathways in that activation leads ultimately to the degradation of the NF κ B inhibitory protein I κ B, allowing nuclear translocation of the transcription factor (O'Connor et al., 2003). Inhibition of NF κ B with the inhibitor Bay11-7082 dose-dependently reduced LPS-induced expression of *Nlrp3* mRNA, suggesting that NLRP3 expression is under the control of the rapid-acting transcription factor (Bauernfeind et al., 2009). As would be expected therefore, pre-treatment of macrophages with Bay11-7082 leads to a reduction in caspase-1 activation (Kahlenberg et al., 2005). As with other levels of the pathways responsible ultimately for the release of IL-1 β and IL-18, induction of NLRP3 expression is regulated in a negative feedback loop whereby the NLRP3 protein inhibits translocation of the p65 subunit of NF κ B to the nucleus. The generation of a series of FLAG-tagged deletion constructs of NLRP3 showed that the NACHT and LRR domains together were responsible for this inhibition (O'Connor et al., 2003). Interestingly co-expression of ASC and NLRP3 has been shown to activate NF κ B, whereas expression of ASC alone inhibited NF κ B activation by stabilising the I κ B-NF κ B complex in TNF- α - and LPS-stimulated transfected cells. Transfection of siRNA targeted at the ASC protein enhanced degradation of I κ B (Stehlik et al., 2002). However another group, using a similar transfection paradigm, found that neither ASC nor

NLRP3 induced NF κ B activation, suggesting that this story is complex and likely depends on cellular context (Yu et al., 2006).

8.4. *Inflammasome activation*

Prior to activator binding, the NLRP3 protein exists in a repressed conformation in the cytosol. Analogous to structurally related plant R proteins, NLRP3, Nod2, and NLRC4 have been found to interact with chaperone proteins SGT1 and hsp90. Further analysis using flag-tagged NLRP3 domains showed that hsp90 binds the NACHT and LRR domains, while SGT1 binds to the LRR domain only. SGT1 binding was found to be reliant on active hsp90; geldanamycin (an inhibitor of hsp90 function) was found to inhibit SGT1-NLRP3 interactions. At larger doses it was reported that geldanamycin substantially reduced endogenous levels of NLRP3, an effect that was prevented by pre-treatment with a proteasome inhibitor. Thus binding of hsp90 appears to prevent proteasome degradation of NLRP3. In contrast, knockdown of SGT1 with siRNA reduced inflammasome activity as assessed by released mature IL-1 β , but did not appear to affect protein stability. Upon binding of an inflammasome activator it is thus postulated that SGT1 and hsp90 dissociate, allowing oligomerisation of the NACHT domains to take place (Mayor et al., 2007). This self-association has been shown to be depend on ATP binding and hydrolysis at the NACHT domain. Transfected cells containing mutated NLRP3 proteins lacking a motif within the nucleotide binding domain of the NACHT domain were shown to produce decreased levels of mature IL-1 β , and NLRP3 was found to self-associate less efficiently than the wt protein. Additionally association of the mutant protein with adaptor protein ASC was drastically reduced, demonstrating that nucleotide binding and hydrolysis is also critically important in recruitment of the adaptor protein (Duncan et al., 2007).

How such a diverse range of structurally distinct activators (particularly in the case of NLRP3) is able to activate the inflammasome is unclear. Each may directly bind to the scaffold protein, or alternatively each ligand may activate a common cellular signal which in turn activates the appropriate inflammasome. Nigericin, maitotoxin, and ATP are all activators of the NLRP3 inflammasome,

and all have been shown to decrease intracellular potassium (Di Virgilio, 2007). It has been shown that addition of ATP to LPS-primed microglial cells causes release of IL-1 β . Active p20 caspase-1 fragment was detected by immunoblot of these cells, and the caspase-1 inhibitor YVAD-CHO prevented this release, showing it to be a caspase-1-dependent mechanism (Sanz and Di Virgilio, 2000). It was postulated that the ATP was acting on P2X7 receptors, a non-selective cation channel, activation of which decreased intracellular potassium concentration. Increased extracellular potassium concentration inhibited release of IL-1 β . Further to this data, Petrilli *et al.* and Dostert *et al.* showed that activation of the NLRP3 inflammasome by a variety of known ligands was inhibited by increasing extracellular potassium concentration, suggesting that potassium efflux is a specific and common trigger to inflammasome activation (Petrilli *et al.*, 2007a, Dostert *et al.*, 2008).

Another common trigger to inflammasome activation appears to be reactive oxygen species (ROS). Increased levels of ROS are reported as a result of incomplete or “frustrated” phagocytosis (Hornung *et al.*, 2008). In the case of asbestos, silica, and MSU activation of the NLRP3 inflammasome, endocytosis and ROS are required to elicit activation (Martinon *et al.*, 2006, Petrilli *et al.*, 2007b, Dostert *et al.*, 2008, Dostert *et al.*, 2009, Gasse *et al.*, 2009). Inhibition of phagocytosis with cytochalasin D prevents NLRP3 activation by crystalline activators as measured by subsequent cytokine release (Dostert *et al.*, 2008, Hornung *et al.*, 2008). Inhibition of ROS by N-acetyl-cysteine (NAC) or (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) pre-treatment has been shown to prevent IL-1 β release in response to asbestos, MSU, and, surprisingly, ATP. Thus ROS may also be a common and necessary trigger for inflammasome activation (Dostert *et al.*, 2008). In a separate study looking at ATP-induced cytokine release in alveolar macrophages, it was shown that P2X7R activation led to a transient production of ROS; detectable within 30 seconds, maximal after 5 minutes, and returned to basal levels 15 minutes after stimulation (Cruz *et al.*, 2007). The P2X7R agonist BzATP was sufficient to cause this production, whereas P2X7R antagonism with oxATP blocked this response. In agreement with Dostert *et al.*, NAC pre-treatment inhibited caspase-1 activation in response to P2X7R activation. Further investigation

revealed that ATP-induced ROS production led to the activation of the Akt and ERK1/2 pathways due to an inhibitory effect on PTEN, the inactivation of which shifts the equilibrium in favour of PI3K activation (upstream of Akt and ERK1/2 pathways). Interestingly inhibition of the PI3K pathway with LY294002 inhibited cytokine secretion in LPS-primed cells stimulated with ATP, and the authors suggest that this pathway is somehow responsible for the activation of caspase-1 (Cruz et al., 2007).

More recently, a yeast two-hybrid screen using the LRR of NLRP3 as bait was employed in order to identify potential NLRP3 binding partners which led to the identification of thioredoxin-interacting protein (TXNIP) as one such protein. Thioredoxin (TRX) acts as an antioxidant which facilitates the reduction of other proteins. TXNIP regulates the antioxidant function of TRX and is thought to act as a negative regulator of the TRX reductase activity. At high concentrations of H₂O₂, TXNIP dissociates from TRX (Zhou et al., 2010). NLRP3 activators R-837 and MSU, which have also been shown to generate ROS, caused TXNIP to dissociate from TRX. This effect was blocked by pre-treatment with the ROS inhibitor APDC. It was shown that MSU-induced dissociation of TXNIP from TRX was maximal one hour after stimulation, at which time association with NLRP3 became evident. siRNA-mediated downregulation of TXNIP suppressed MSU-, R-837-, and ATP-induced cytokine release. The authors proposed a model whereby TXNIP dissociates from TRX following its oxidation by ROS, allowing TXNIP to bind to, and participate in the activation of, NLRP3 (Zhou et al., 2010).

It was initially posited that the source of the ROS might be the phagosomal respiratory-burst oxidase system, associated with the degradation of internalised bacterial particles. However, normal NLRP3 inflammasome activation in response to silica crystals, MSU, and ATP occurs in mice lacking gp91phox, a subunit of the phagosomal NADPH oxidase cytochrome b (Hornung et al., 2008). The main source of cellular ROS is the mitochondria, which has prompted investigation into the role of mitochondrial ROS in NLRP3 inflammasome activation. Treatment of human THP-1 macrophages with rotenone, a complex I inhibitor, or antimycin, a complex III inhibitor, led to IL-1 β

secretion in a dose-dependent manner, which was prevented when cells were pre-treated with the ROS inhibitor APDC. This effect was not observed in macrophages obtained from mice deficient in NLRP3 (Zhou et al., 2011). Inhibition of mitophagy, which leads to an accumulation of damaged mitochondria and thus increased concentrations of mitochondrial ROS, led to the enhanced secretion of IL-1 β (Nakahira et al., 2011, Zhou et al., 2011). Mice lacking LC3B, a protein contributing to the process of autophagy, were shown to produce more IL-1 β and IL-18 in a model of septic shock than wildtype littermates (Nakahira et al., 2011). Finally inhibition of voltage-dependent anion channels (VDAC) VDAC1 and VDAC2 (major channels for the exchange of metabolites and ions between the mitochondria and other cellular compartments) caused a significant reduction of IL-1 β secretion in response to NLRP3 activators. However VDAC3 inhibition did not affect inflammasome activity (Zhou et al., 2011). Further investigation into the role of ROS in inflammasome activation has led one group to conclude that rather than directly activating NLRP3, ROS function to upregulate NLRP3 expression. This conclusion was made on the basis that ROS inhibition decreased the LPS-mediated induction of IL- β , TNF α , and NLRP3. Cells treated with ROS inhibitors after the LPS-priming step still showed NLRP3 activation and cytokine secretion in response to nigericin. In support of this hypothesis, ROS inhibition in cells stably overexpressing NLRP3 did not affect NLRP3 activation (Bauernfeind et al., 2011).

In addition to K⁺ depletion and the action of ROS, the protease cathepsin B has been proposed to play a role in inflammasome activation in response to the crystalline and particulate activators. It was proposed that phagocytosis of crystals leads to destabilisation and rupture of lysosomes, leading to the release of, among others, the protease cathepsin B. In line with this hypothesis, pre-treatment of cells with the inhibitor CA-074-Me led to reduced secretion of IL-1 β following stimulation with silica, or amyloid beta (Halle et al., 2008, Hornung et al., 2008). However, macrophages from mice deficient in cathepsin B showed normal NLRP3 activation in response to hemazoin, MSU, and alum (Dostert et al., 2009). This suggests an off-target effect of CA-074-Me in the other reports, possibly acting to inhibit another protease released

from damaged lysosomes. A schematic representing the activation of the NLRP3 inflammasome is shown in Figure i.8.

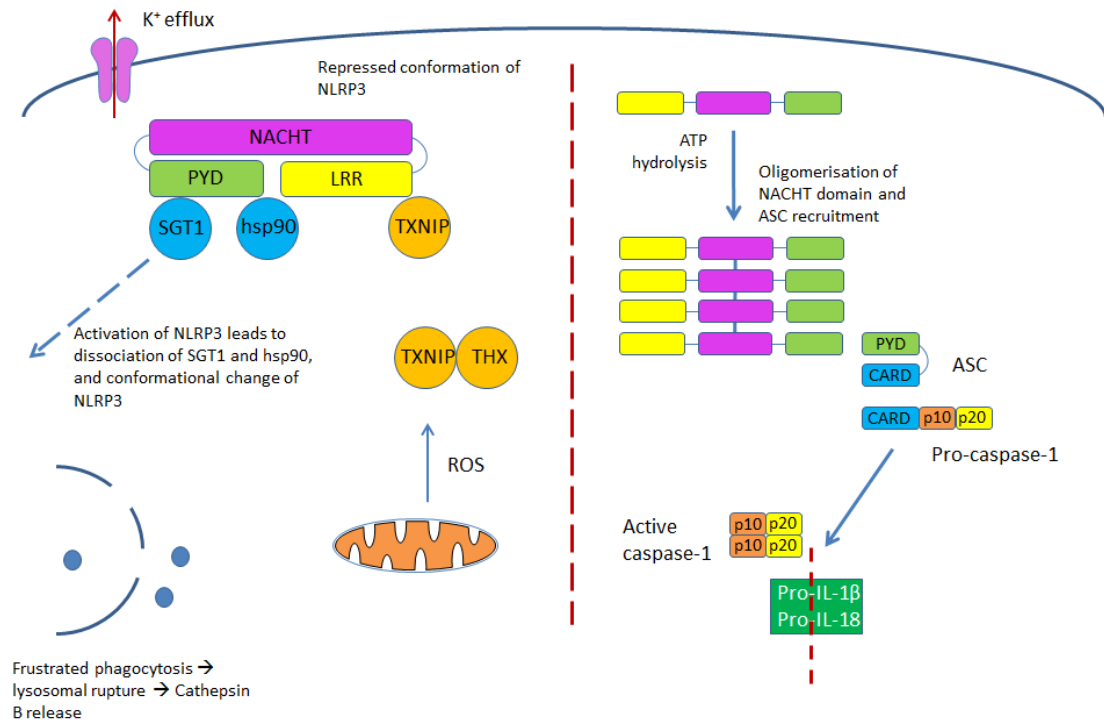


Figure i.8: Overview of NLRP3 inflammasome activation

NLRP3 exists in a repressed state, held in an inactive conformation by chaperone proteins SGT1 and hsp90. NLRP3 is activated by a range of molecules; it is postulated that common, downstream, pathways activate NLRP3. Putative activators include K⁺ efflux, possibly via P2X7 receptor activation, Cathepsin B released from lysosomes, and mitochondrial ROS. Increased ROS levels result in the dissociation of TXNIP from binding partner THX, and binds the LRR of NLRP3. Once activated SGT1 and hsp90 dissociate and are degraded, resulting in a change in NLRP3 conformation. ATP binding and hydrolysis at the NACHT domain results in oligomerisation at the NACHT domain and recruitment of adaptor protein ASC, via PYD-PYD protein interactions. Pro-caspase-1 is recruited to the complex via CARD-CARD interactions. Induced proximity results in the autocleavage of pro-caspase-1 generating active caspase-1 tetramers, consisting of two p10 and two p20 subunits. Active caspase-1 dissociates from the complex and goes on to cleave pro-IL-1 β and pro-IL-18.

8.5. Abnormal inflammasome signalling in disease

IL-1 β is a pro-inflammatory cytokine with a range of physiological effects, including generation of fever, rash, and the upregulation of the expression of other pro-inflammatory cytokines. IL-1 β also appears to have homeostatic functions including the regulation of feeding, sleep, and temperature (Ren and Torres, 2009). Thus it is hardly surprising that there exist multiple levels of control in the expression, maturation, and release of this cytokine. The need for so many levels of regulation is exemplified in several autoimmune disorders, characterised by an excessive and uncontrolled release of IL-1 β . Cryopyrin-associated periodic syndromes (CAPS) are hereditary diseases characterised by recurrent fever and inflammation often with a lack of detectable cause. Symptoms include rash, fever/chills, joint and muscle pain. There are three CAPS syndromes; familial cold autoinflammatory syndrome (FACS), Muckle-Wells syndrome, and chronic infantile cutaneous neurological articular syndrome (CINCA). Macrophages from patients with Muckle-Wells syndrome have been shown to spontaneously secrete IL-1 β (Agostini et al., 2004). CAPS patients respond well to treatment with IL-1RA. CAPS syndromes have been shown to be caused by mutations in the gene encoding the NLRP3 protein, specifically in exon 3 which encodes the NACHT domain (Hoffman et al., 2001, Feldmann et al., 2002).

Gout is an autoinflammatory disease characterised by severe joint inflammation. Gout is caused by elevated levels of uric acid in the blood. The uric acid crystallises to form monosodium urate (MSU), and becomes deposited in joints, tendons, and muscles. MSU is a potent activator of the NLRP3 inflammasome (Martinon et al., 2006).

Elevated IL-1 β has also been identified as a risk factor in the development of type II diabetes as it has been shown to mediate the toxic effects of prolonged hyperglycaemia in pancreatic islets (Schroder and Tschopp, 2010). It has been shown that high glucose concentrations induce IL-1 β secretion. This effect is considerably reduced in islets from mice deficient in TXNIP, and from mice deficient in NLRP3, suggesting that high glucose concentration activates the inflammasome in islet cells. In line with this mice deficient in TXNIP and

NLRP3 showed improved glucose tolerance and insulin sensitivity (Zhou et al., 2010).

8.6. A role for the NLRP3 inflammasome in pain?

There are several lines of evidence which might indicate a role of the inflammasome in both neuropathic and inflammatory pain states. IL-1 β is algogenic when administered to a variety of tissues, and its expression has been shown to be upregulated in several animal models of pain, including CCI and intraplantar injection of CFA (Fukuoka et al., 1994, Cunha et al., 2000, Samad et al., 2001, Sung et al., 2004, Kawasaki et al., 2008b, Gabay et al., 2011). As previously mentioned, microglia and macrophages, which express and release IL-1 β , have been strongly implicated as having a role in the development of pain-like behaviours in several animal models of pain. To date there is little investigation of the inflammasome in microglia, though one publication has investigated the role of the inflammasome in the microglial response to amyloid-beta (Halle et al., 2008), demonstrating a presence of the inflammasome within microglia. IL-1 β , as well as activated caspase-1 and ASC, have been shown to be released from LPS-stimulated *ex-vivo* dorsal horn slices. This effect is inhibited by treatment with a glial inhibitor (Clark et al., 2006). P2X7 receptor activation has been shown to cause inflammasome activation in primed cells, and, as discussed earlier, P2X7 receptor signalling plays a role in pain signalling (Chessell et al., 2005, Honore et al., 2009, Clark et al., 2010b). TLR4 signalling in microglia has been shown to play a role in the initiation of neuropathic pain-like behaviours in mice (Tanga et al., 2005), and, as discussed above, TLR signalling converges to activate transcription factor NF κ B which, in some cellular contexts at least, upregulates pro-IL-1 β and NLRP3 expression (Bauernfeind et al., 2009, Berger et al., 2011). Finally it has been shown that ROS production in animal models of neuropathic and inflammatory pain contribute to behavioural hypersensitivity (van de Loo et al., 2003, Gao et al., 2007, Ibi et al., 2008, Kim et al., 2010a, Berger et al., 2011). Pre-treatment with the antioxidant sulforaphane reduced pain-like behaviours, microglial activation, and expression of pro-inflammatory cytokines TNF- α and IL-1 β following SNT injury. Nox2 was found to be upregulated, and mice

deficient in Nox2 were shown to have attenuated ROS activation following SNT injury compared to wildtype litter mates (Kim et al., 2010a).

Thus the aim of the research presented in this thesis was to investigate a possible role for the NLRP3 inflammasome, upstream of IL-1 β maturation and release, in animal models of chronic pain.

Chapter 1: Inflammasome Expression in Microglia and Macrophages, and its Dysregulation Following Peripheral Nerve Injury

1. Introduction

Neuropathic pain, a debilitating condition, arises as a consequence of neural injury with symptoms such as spontaneous pain, hyperalgesia, and allodynia commonly reported. The underlying mechanisms involved in the generation of such pain syndromes are currently unclear. In several animal models of neuropathic pain it has been shown that there is accumulation and proliferation of microglial cells in the injured spinal cord and macrophages within injured nerve and dorsal root ganglion (DRG), contributing to the development of pain-like behaviours (Hu and McLachlan, 2002, Tsuda et al., 2005, McMahon and Malcangio, 2009, Calvo et al., 2012). In studies in which microglial activation was prevented, for instance with minocycline, the development of pain-like behaviours in response to nerve injury was also prevented, demonstrating the importance of microglial contribution in generating pain states (Raghavendra et al., 2003, Ledeboer et al., 2005, Sun et al., 2012b). Microglia are the resident immune cells of the CNS, and share many properties with the phagocytic macrophage. Under normal physiological conditions microglia are thought to act to stabilise the CNS by surveying the local environment for stimuli and changes which might indicate a threat to the physiological homeostasis of the system such as trauma, ischaemia, and infection. Insult to the CNS causes microglia to undergo a series of phenotypic changes including changes in gene expression, cell proliferation, migration to the site of injury or threat, and secretion of pro-inflammatory molecules such as TNF α , NO, IL-18 and IL-1 β (Ransohoff and Perry, 2009). The precise role of macrophages in the DRG following peripheral nerve injury is not fully understood, though it is likely that they release inflammatory mediators such as Cathepsin S, TNF- α , and IL-1 β , which sensitise sensory neurons (Sommer and Kress, 2004, Ozaktay et al., 2006, Barclay et al., 2007). Depletion of macrophages has been found to reduce peripheral nerve injury-induced behavioural hypersensitivity (Liu et al.,

2000a), demonstrating the importance of macrophage contribution in generating pain states.

The pro-inflammatory cytokine IL-1 β plays a key role in acute and chronic inflammation. It has been shown to cause mechanical and thermal hyperalgesia when injected into peripheral tissues, and increased expression of IL-1 β in the spinal cord, DRG, and injured nerve is seen in several animal models of neuropathic pain (Ren and Torres, 2009). IL-1 β is synthesised as an inactive pre-cursor, pro-IL-1 β , that must be enzymatically cleaved by active caspase-1 to generate mature active IL-1 β . The inflammasome is a multi-protein complex which acts as a regulating platform for the eventual cleavage and secretion of mature IL-1 β via activation of caspase-1. Caspase-1 also cleaves pro-IL-18, to generate the mature cytokine IL-18 (Fantuzzi et al., 1999). IL-18 has also, more recently, been shown to potentially play a role in animal models of pain (Miyoshi et al., 2008). The inflammasome complex consists of a central scaffold protein for which it is named (eg NLRP1, NLRP3, NLRC4), an adaptor protein, ASC, and pro-caspase-1. Inflammasome complexes assemble in response to a variety of exogenous and endogenous danger signals (eg MSU crystals, ATP, flagellin) (Schroder and Tschopp, 2010). Of the inflammasome complexes described to date, the NLRP3 inflammasome appears to have the most restricted tissue distribution pattern, found mainly in immune cells (Kummer et al., 2007). The NLRP3 protein consists of three domains; a leucine rich region (LRR), the putative agonist-binding region, the NACHT domain, and a PYRIN domain. Upon activation the inflammasome assembles by oligomerisation of the scaffold at the NACHT domain. The adaptor protein ASC, which contains a PYRIN and CARD domain, is recruited to the complex via PYRIN-PYRIN domain protein interactions. In turn, inactive pro-caspase-1 is recruited to the complex via CARD-CARD protein interactions with the adaptor protein ASC. Autolysis of pro-caspase-1 to the p10/p20 active caspase-1 tetramer is then able to occur due to the clustering induced by recruitment to the complex. Active caspase-1 processes pro-IL-1 β and pro-IL-18 to generate the active cytokines which are subsequently released, along with caspase-1, via non-classical secretory pathways.

The inflammasome, part of the innate immune system, is best studied in the field of immunology. The NLRP3 inflammasome is expressed in murine macrophages, and these cell types are commonly employed to study inflammasome responses. It is only more recently that a potential role for the inflammasome in CNS pathologies, such as Alzheimer's disease, Parkinson's disease, and Multiple Sclerosis, has gained attention (Halle et al., 2008, Chakraborty et al., 2010). This study set out to determine if the NLRP3 inflammasome, known to be expressed in macrophages, is also expressed in microglia. This study also sought to determine if the inflammasome is activated following L5 spinal nerve transection injury, a commonly used animal model of neuropathic pain in which microglia and macrophages are thought to contribute to pain-like behaviours.

2. Methods

2.1. *Primary microglia culture*

Primary microglial cultures were prepared using post-natal day (P)3-5 Wistar pups, killed by decapitation. The brains were removed and the meninges dissected away. The tissue was minced and digested with EDTA Trypsin (Invitrogen) for 30 minutes at 37°C, 5% CO₂. Trypsination was stopped with addition of Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 15% foetal bovine serum (FBS), 1% penicillin/streptomycin, and 100µl DNAase1 (Sigma, 150U/10ml). The tissue was thoroughly mixed. The tissue clumps were allowed to settle and the supernatant removed and filtered through a 80µm mesh. This process was repeated a further two times. The collected supernatant was then centrifuged at 2250 g for 5 minutes. The resulting supernatant was discarded and the cells re-suspended in DMEM + 15% FBS, and plated in 75ml², seeded at 4-5 million cells per flask.

Cultures were maintained at 37°C, 5% CO₂. Media was changed every 2-3 days. After 5 days in vitro the flasks were confluent with astrocytes and microglia. Flasks were shaken by hand for 5 minutes in order to detach the microglia (formed in a monolayer above the astrocytes), and the media removed and centrifuged at 2250 g for 5 minutes. The supernatant was discarded and the cells re-suspended, ready for experimental use.

For Western blotting experiments, cells were plated in 6 well plates at a density of 1×10^6 cells per well. 15-20 minutes after plating the medium was changed to discard any non-adherent cells. Cells were then incubated overnight to allow them to become more "resting". In the morning the media was removed, the cells washed in HBSS in order to ensure all traces of FBS were removed, and then incubated in serum-free media for 3 hours before stimulation protocols were carried out to ensure microglia were in a resting state before experimentation. For immunocytochemistry and ELISA experiments cells were plated in 24 well plates at a density of 5×10^4 cells per well, on sterile glass coverslips. The same protocol as above was followed.

2.2. *Peritoneal macrophage culture*

Adult female Wistar rats were killed with increasing CO₂ concentrations. Immediately after they were injected intraperitoneally with 40ml of ice-cold sterile Hank's Balanced Salt Solution (HBSS), and the area gently massaged. The elicited peritoneal exudate cells were harvested and re-suspended in DMEM supplemented with 15% FBS, 1% penicillin/streptomycin. Media was changed every 2-3 days.

As for the microglia, cells used for Western blot experiments were plated at a density of 1×10^6 cells per well in a 6 well plate and incubated at 37°C, 5% CO₂. Media was changed 15-20 minutes after plating to discard any non-adherent cells. Thereafter media was changed every 2-3 days. Cells were used for LPS/ATP stimulation experiments one week later, when the cells had taken on a completely resting morphology (ramified processes, not "bright" and round in appearance as at time of culture). On the day of experimental use, media was removed and the cells were washed in HBSS in order to ensure all traces of FBS were removed, and then incubated in serum-free media for 3 hours before stimulation protocols were carried out to ensure cells were in a resting state before experimentation. For immunocytochemistry experiments cells were plated in 24 well plates at a density of 5×10^4 cells per well, on sterile glass coverslips. Media were changed 15-20 minutes later to discard any non-adherent cells. Media were thereafter changed every 2-3 days. Cells were typically ready for experimentation 3-4 days after culture. On the day of use the same protocol as above was used.

2.3. *Immunocytochemistry*

Microglial and macrophage cells were plated at a density of 50,000 cells per well on a 24-well plate. Cells were fixed for 10 min with 100% ice-cold methanol, followed by 30 min with 4% ice-cold paraformaldehyde (PFA). Plates were stored at 4°C until required. Wells were washed 3 times with PBS. Antibody block was not performed. Primary antibodies were applied for 3 hours, followed by washing with PBS as above. Secondary antibodies were

then applied for 1.5 hours, followed by washing with PBS as above. Microglia were labelled with biotin-conjugated isolectin B4 (IB4) (1:50, Sigma), and visualised with Extravidin FITC (1:200, Sigma). Macrophages were labelled with rabbit anti-IBA1 (1:1000, WAKO), and visualised with Alexa Fluor 488 (1:1000, Stratech). As a negative control, antibody specificity was confirmed by the omission of primary antibody (data not shown). Coverslips were mounted onto glass slides using Vectashield mounting medium with DAPI (Vectorlabs) and visualised using a Zeiss Axioplan 2 fluorescent microscope. Culture purity of 5 separate cultures was assessed by taking 10x images of 3 random fields per slide. The number of IB4 / IBA1 positive cells was divided by the number of DAPI stained nuclei and multiplied by 100 to give a percent purity.

2.4. Western blot

Cell stimulation: Harvested microglial cells, and primary macrophage cells were plated at a density of 1×10^6 cells per well in a 6 well plate and allowed time to "rest". Media were replaced with serum-free media for 3 hours before stimulation with LPS (1 μ g/ml) or vehicle for 4 hours, followed by ATP (5mM) or vehicle for 15 minutes. Each experiment was carried out 3 times, using 3 separate cultures.

Media: Media were collected and stored at -80°C. Proteins were precipitated using trichloroacetic acid (TCA). Briefly samples were incubated with 1:5 TCA stock at 4°C for 10 minutes and then centrifuged at 100,000 g 4°C for 5 minutes. Carefully supernatant was removed and remaining cell pellets were washed with acetone twice before being dried for 5-10 minutes at 95°C. Pellets were re-suspended first in 10 μ l 0.2M NaOH, in order to aid re-suspension of the pellet, and then in 30 μ l lysis buffer.

Cells: Cells were lysed in 80 μ l lysate buffer (20mM Tris, pH8, 137mM NaCl, 1%NO-40, 1mM sodium orthovanadate, and protease inhibitor mixture (Pierce, containing 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, E-64, leupeptin, pepstatin, and EDTA)). Cells were scraped, and the lysates removed. Lysates were centrifuged at 100,000 g at 4°C for 15 minutes, and supernatant stored at -80°C.

Collected animal tissue: Tissues were homogenised in lysis buffer (as above), and rotated gently at 4°C for two hours. Samples were then centrifuged at 100,000 g for 15 minutes. The supernatant was stored at -80°C, while the pellet was discarded. Numbers of animals used differed between experiments. Refer to individual study results for this information.

Protein concentration of all samples was determined using a BCA Protein Assay kit (Thermo Scientific). Cell and tissue lysates were normalised to 35 µg protein per sample. Although media sample protein concentrations were determined, they were not normalised owing to the assumption that LPS/ATP stimulated cells would have released more protein into the extracellular media than control-treated cells. Normalisation would have diluted any observed effect of protein secretion. Proteins (35 µg cell lysate, or 40 µl resuspended media protein solution [see **Media**, above] per well) were separated using SDS-PAGE (15% gel to visualise ASC, caspase-1, 10% to visualise NLRP3) and transferred to PVDF membranes (Millipore) at 100 V for 60 minutes. For ASC and Caspase-1 blots, PVDF membranes of 0.2 µm pore size were utilised (Millipore). Membranes were blocked in 10% skimmed milk in PBS-T for 45 minutes before overnight incubation with primary antibody at 4°C. The membranes were then washed for 5 minutes, 6 times in PBS-T before incubation with species appropriate HRP-conjugated secondary antibody (1:10,000, Amersham) for 1 hour at room temperature. Membranes were visualised using ECL-prime reagent for 5 min (GE Healthcare) for detection by autoradiography.

Membranes were stripped with a 50°C pre-heated β-mecaptoethanol-containing buffer (0.7% β-mecaptoethanol, 20% 10% SDS, 6% 1M Tris-HCl pH 6.7), incubated for 30 min at 50°C. Cells were then washed thoroughly first in dH₂O, then several times in PBS-T, as above. Membranes were blocked in 10% skimmed milk in PBS-T for 45 minutes, before incubation with primary antibody for 1 hour. The membrane was washed in PBS-T, as above, before incubation with species appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. Membranes were visualised as above.

Antibodies used: Rabbit anti-caspase-1 (1:400, Abcam, antibody recognises p20 subunit of active caspase-1, and p45 subunit of pro-caspase-1), rabbit anti-ASC (1:1000, Alexis Biochemicals), NLRP3 (0.25 µg/ml, Adipogen), mouse β -actin (1:15,000, Sigma), donkey anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:20,000, GE Healthcare), donkey anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:20,000, GE Healthcare).

Blots were analysed by determining mean intensity of each band using a fixed box size for all. The background intensity for each blot was then subtracted from all values obtained. All quantifications were then normalised against β -actin loading control. All blots were then compared to the mean band intensity for control-treated samples or naive animals. Analysis was carried out using Quantity One 1-D Analysis Software version 4.6.9 (Biorad).

2.5. *IL-1 β ELISA*

Harvested microglial cells were plated on sterile glass coverslips at a density of 5×10^4 cells per well in 24-well plates, and left overnight. Cells were washed in HBSS, and then media was replaced with serum-free media for 3 hours before stimulation with LPS (1 µg/ml) or vehicle for 4 hours, then ATP (5 mM) or vehicle for 15 minutes. Media were collected for ELISA analysis using Quanktikine IL-1 β ELISA kit (R&D Systems). Kit-supplied standards were run in triplicate, and test samples in duplicate following the manufacturer instructions. Each experimental condition was performed in triplicate (i.e. 3 wells for control, 3 wells for LPS, etc.). The optical density data for each triplicate were averaged. The experiment was performed on 3 separate occasions using 3 separate microglial cultures. Averaged triplicate data from each experimental set were combined and meaned to give an overall result. All samples were tested using the same ELISA kit to eliminate inter-experiment variability. Samples were read at 450 nm with wavelength correction at 540 nm.

2.6. *Animals and surgery*

Adult male Wistar rats (Harlan, UK) were used in accordance with UK Home Office regulations (Animals Scientific Procedures Act 1986). Animals were anaesthetised using a mixture of medetomidine hydrochloride (0.25 mg/kg) and ketamine (60 mg/kg) administered through a single intraperitoneal injection. The left L5 spinal nerve was exposed by removing the relevant vertebral process, ligated with 6-0 silk suture for later identification, and transected distal to the suture. The muscle layer was sutured, and the wound closed with a Vicryl 3-0 suture. In animals undergoing “sham” surgery, the L5 spinal nerve was exposed in the same way, but was not transected. The muscle layer was sutured and the wound closed in the same way as the other animals. No post-surgical analgesia was given in order to avoid potential modulation of the nociceptive and immune systems of the animals. The general health of the animals was monitored daily after the surgery.

Animals were sacrificed by terminal anaesthesia and transcardially perfused with either 4% paraformaldehyde plus 1.5% picric acid for histology, or saline alone for Western blot analysis at defined survival times. Tissue used for histology: Lumbar spinal cords were excised and post-fixed overnight, dorsal root ganglia (DRG) were post-fixed for 3-4 hours. Tissue was cryoprotected in 20% sucrose in 0.1M Phosphate Buffer (PB) for 4-7 days before OCT embedding. Tissue used for Western blot analysis: Lumbar spinal cords were excised and quadrisectioned. The ipsilateral dorsal horns were used for Western blot analysis. Lumbar spinal cords and DRG were snap-frozen in liquid nitrogen and stored at -80°C.

2.7. Behavioural testing

Animals were tested in a temperature stable room during the light period of their day/night cycle, at the same time every day. Animals were placed in a polymethyl methacrylate box with a wire grid bottom and allowed to habituate for a period of 15-30 minutes before testing. Animals were trained once for each behavioural test, and then two baseline tests were performed on different days.

Assessment of mechanical withdrawal thresholds: Mechanical withdrawal thresholds were assessed using calibrated von Frey hairs (Ugo Basile) according to the “up-down” method (Chaplan et al., 1994). Briefly, nylon filaments were applied to the plantar surface of the hindpaw until they just bent and were held in position for 5 seconds. A positive response was defined as rapid withdrawal of the paw not associated with other behaviours such as locomotion or grooming. In the event of a positive response, the next lower hair was applied until a change in response was observed. Conversely in the event of a negative response, the next higher hair was applied until a change in response was observed. Four subsequent hairs were then assessed using this up/down sequence. The 50% withdrawal threshold was then calculated using the method described by Dixon (Dixon, 1980).

Cold testing: For measurement of cold hypersensitivity, a drop of acetone was applied to the plantar surface of the rat hindpaw. Evaporation of the acetone exerts a cooling effect to which naïve animals do not normally respond. However nerve injured animals respond by licking and shaking the paw. The time spent behaving in this manner was measured in the 2 minutes following acetone application. 3 tests were carried out, at least 10 minutes apart from each other, and an average response was recorded.

2.8. Histology

Lumbar spinal cords were cryostat cut in 20 µm sections and thaw-mounted onto glass slides. When slides were fully dry (at least 1 hour after tissue mounting), they were stored in cryoprotectant (30% ethylene glycol, 30% glycerol, 40% PBS) at -20°C until required.

Tyramide Signal Amplification: Slides were washed for 5 minutes, 6 times, in PBS to ensure all OCT and cryoprotectant was washed away from the sections. Sections were incubated for 30 minutes with 10% normal goat serum in PBS, 0.2% Triton X, 0.1% Azide, and 1% H₂O₂, to quench any peroxidases, followed by overnight incubation with primary antibody. The signal was then amplified according to kit manufacturer instructions (TSA Biotin System, Perkin

Elmer). Briefly, slides were incubated with species appropriate biotinylated antibody (1:400, Vectorlabs) for 1.5 hours. Slides were then washed for 5 minutes, 3 times, in PBS. ABC solution (Vectorlabs ABC kit), made 30 minutes in advance, was applied to the sections for 30 minutes. Slides were then washed as before. Biotinyl tyramide solution (1:50, TSA Biotin System, Perkin Elmer), was then applied for 10 minutes. Slides were washed as before. Finally sections were incubated with ExtrAvidin FITC (1:200, Sigma) for 2-3 hours. Slides were washed as before. Slides were then either mounted on coverslips, or subjected to a conventional staining protocol for co-staining purposes.

Conventional immunohistochemistry: Slides were washed, as above. Sections were incubated for 30 minutes with 10% normal donkey serum in PBS, 0.2% Triton X, 0.1% Azide, followed by overnight incubation with primary antibody. The slides were washed, as before, and incubated in species appropriate secondary fluorescent antibody for 2-3 hours. Slides were washed and mounted on coverslips using Vectashield mounting medium with DAPI (Vectorlabs) and visualised using a Zeiss Axioplan 2 fluorescent microscope. Specificity of staining was determined by omission of the appropriate primary antibody as a negative control.

Primary antibodies: The primary antibodies ASC and caspase-1 were visualised using tyramide amplification. Rabbit anti-ASC (1:2000, Alexis Biochemicals), rabbit anti-caspase-1 (1:500, Abcam), mouse anti-NeuN (1:100, Chemicon), rabbit anti-GFAP (1:1000, DAKO), rabbit anti-IBA1 (1:1000, WAKO). Secondary antibodies: anti-mouse or anti-rabbit Alexa Fluor 488 (1:1000, Stratech), anti-mouse or anti-rabbit Alexa Fluor 546 (1:1000, Stratech), ExtrAvidin FITC (1:200, Sigma).

Quantification:

Cell counts / colocalisation: 3 100 μm^2 boxes were drawn over the superficial L5 dorsal horn, ipsilateral and contralateral to the injury. Cells which were associated with a nuclear stain, within the confines of the box, were counted. Counting was performed manually. An average count from the 3 boxes was

taken. At least 3 sections per animal were analysed. All analysis was carried out blind to treatment.

2.9. qPCR

This body of work was kindly carried out in collaboration with Dr. Ana Antunes-Martins.

RNA extraction: DRG and ipsilateral lumbar dorsal horn tissue samples were flash frozen in liquid nitrogen immediately after dissection. Total RNA was extracted using the miRNeasy mini kit (Qiagen), according to manufacturer's instructions, including optional treatment with RNase-free DNase (Qiagen). RNA quantity and purity were measured with a NanoDrop spectrophotometer 2000 (Thermo Scientific Inc.) and integrity was assessed using Agilent RNA 6000 Pico chips (Agilent) on an Agilent 2100 Expert Bioanalyzer.

Quantitative real-time PCR: Total RNA was reverse transcribed into double stranded cDNA using random primers (Promega) and Superscript III Reverse Transcriptase (Invitrogen). cDNA QPCR amplification was performed using the LC FS DNA MasterPLUS SG (Roche), with final primer concentrations of 1 μ M. Amplifications were performed in a Rotor Gene 6000 (Corbett Research) instrument, with the following conditions: 10 min at 95°C and 40 cycles of 10 sec at 95°C, 10 sec at 60°C, 20 sec at 72°C. Relative Quantification to the housekeeping gene GAPDH was performed by the Rotor Gene 6000 software (v1.7) using the DeltaDelta Ct method. Primer pairs used were:

Gene	Direction	Sequence (5' > 3')	Efficiency
Caspase-1	Forward	CCGACAAGGTCCTGAGGGCAA	1.17 (R2=0.99242)
	Reverse	TCGCACAGGTCTCGTGCCTT	
NLRP3	Forward	ACCCAAGCCCTACTGTTCT	1.05 (R2=0.99190)
	Reverse	TCAGGTCACCAAGAGGGAAC	

2.10. Statistical analysis

All data are represented as mean \pm SEM where appropriate. ELISA, Western blot, and histology data were analysed using one-way ANOVA followed by Dunnett's post-test. Behavioural data were analysed using two-way repeated

measures ANOVA followed by Dunnett's post-test. Where data were not normally distributed, ANOVA tests were performed on ranked data. All analysis was carried out using the statistical package SigmaPlot for Windows, version 12.

3. Results

3.1. *Primary cultures are >90% pure*

Primary rat microglial cultures were found to be, on average, $95.29 \pm 0.79\%$ pure when assessed in five separate experiments. Primary rat peritoneal macrophage cultures were found to be, on average, $94.44 \pm 2.14\%$ pure when assessed in three separate experiments. The number of IB4-(microglia) or IBA1-positive (macrophages) and negative cells were counted to assess the purity of the cultures. 3 random field images were taken of cells from at least 3 mounted coverslips. Morphologically, microglia had long, thin processes with small cell bodies, indicating a “resting” phenotype. Macrophages similarly had small cell bodies, with some branching processes, though less than those seen in microglia. Representative images are shown in Figure 1.1 a and b.

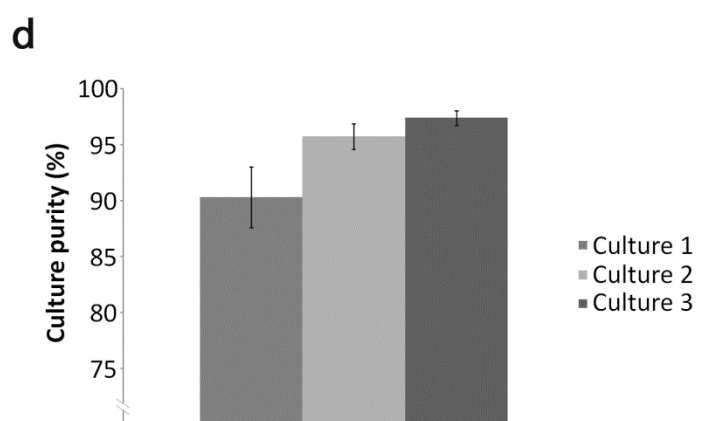
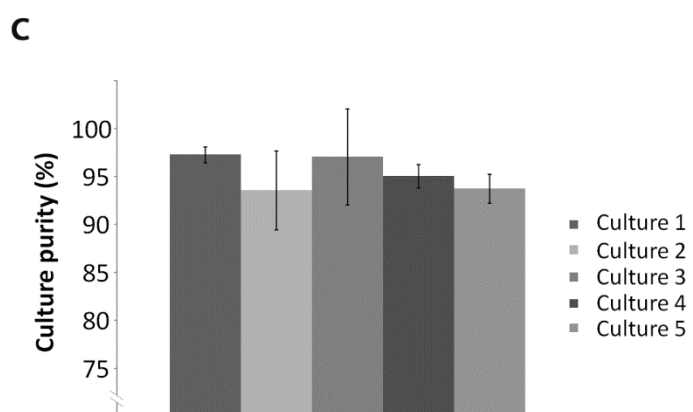
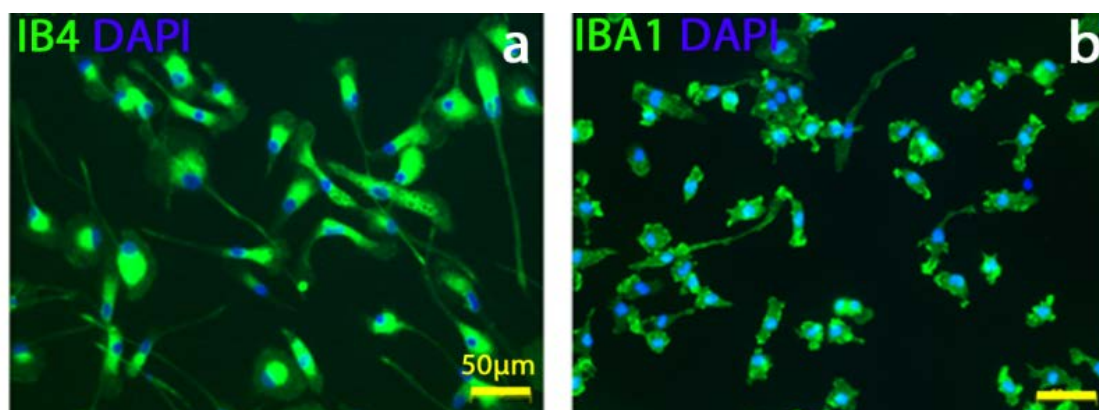


Figure 1.1: (legend on the next page)

Figure 1.1: Primary cultured microglia and primary cultured peritoneal macrophages

a: Representative image of rat primary cultured microglia. IB4 represented in the green channel, DAPI represented in the blue channel. Scale 50 μm . **b:** Representative image of rat primary cultured peritoneal macrophages. IBA1 represented in the green channel, DAPI represented in the blue channel. Scale 50 μm . **c:** Graph shows the average purity of 5 different microglial cultures. Data represented as mean \pm SEM. **d:** Graph shows the average purity of 3 different macrophage cultures. Data represented as mean \pm SEM.

3.2. Microglia express the NLRP3 inflammasome

Components of the NLRP3 inflammasome are known to be highly expressed in macrophages, and more recently have been shown to be present in microglia (Halle et al., 2008). Cultured rat peritoneal macrophages and postnatal microglia were used to confirm expression of NLRP3 inflammasome components and sensitivity of IL-1 β release to caspase-1 inhibition in these cells. Consistent with previous reports (Qu et al., 2007) the component proteins of the NLRP3 inflammasome NLRP3, ASC, and caspase-1 were found to be expressed in rat peritoneal macrophages (Figure 1.2 a-c). Cells were primed with LPS (1 μ g/ml) for 4 hours before being stimulated with ATP (5mM), a well-characterised activator of the NLRP3 inflammasome, for 15 minutes. This protocol was chosen in line with that published by other groups using LPS/ATP stimulation of macrophages to study inflammasome activation (Sanz and Di Virgilio, 2000, Mariathasan et al., 2004, Qu et al., 2007). Immunoblot of cell lysates showed a significant increase in NLRP3 protein (7.62-fold \pm 2.19, $p < 0.05$ compared to unstimulated control), which was almost undetectable in unstimulated cells. NLRP3 protein was not detected in extracellular media from either control or stimulated cells. Immunoblot of extracellular media taken from stimulated cells showed significantly increased levels of ASC and the 20kDa active form of caspase-1 compared to unstimulated cells (9.12-fold \pm 4.44, $p < 0.05$, and 2.12-fold \pm 0.22, $p < 0.01$ respectively).

NLRP3 inflammasome components were also found to be present in postnatal cultured microglia (Figure 1.2 d-f). Similar to the data shown in macrophages, ATP stimulation of LPS-primed microglia led to a significant increase in NLRP3 protein in cell lysates compared to unstimulated control cells (2.53-fold \pm 0.48, $p < 0.05$), and a small but significant decrease in ASC protein (0.70-fold \pm 0.11, $p < 0.05$), likely reflecting ejection of the protein with active caspase-1 and IL-1 β . Immunoblot of extracellular media showed a significant increase in ASC and the 20kDa active form of caspase-1 protein compared to that of unstimulated control cells (4.3-fold \pm 0.92, $p < 0.01$, 2.80 \pm 0.65, $p < 0.05$ respectively). ATP stimulation of LPS-primed microglia resulted in IL-1 β release into the extracellular media (1207.25 \pm 39.01 pg/ml), an endpoint in the

inflammasome activation pathway (Figure 1.2 g). To determine if this effect was caspase-1 dependent cells were pre-treated with 1- and 10 μ M Ac-YVAD-CMK, a cell-permeant peptide inhibitor of caspase-1, for 2 hours prior to ATP stimulation. YVAD dose-dependently decreased the amount of IL-1 β released from stimulated cells (1007.12 ± 42.02 pg/ml, $p < 0.05$, and 630.18 ± 54.81 pg/ml, $p < 0.001$ compared to LPS/ATP stimulation respectively).

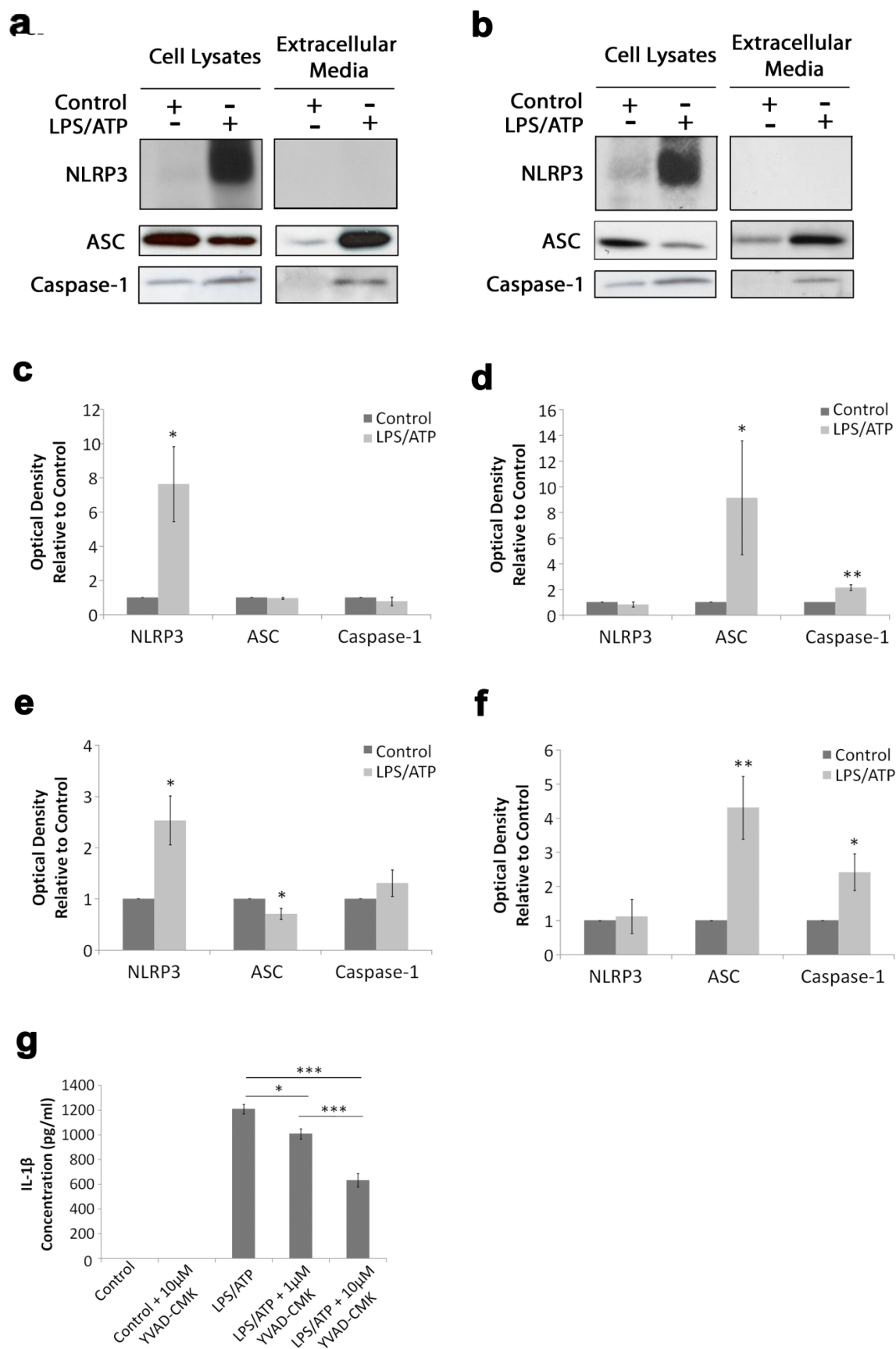


Figure 1.2: (figure legend on the next page)

Figure 1.2: The NLRP3 inflammasome is expressed, and can be activated, in primary peritoneal macrophages and primary microglia

Western blot analysis shows primary peritoneal macrophages express inflammasome components NLRP3, ASC, and active caspase-1 p20 subunit under control, non-stimulated conditions. ATP stimulation of LPS-primed cells alters expression of inflammasome components in cell lysates and extracellular media, quantified in **c** and **d** respectively. Representative blots shown in **a**. Primary microglia also express inflammasome components NLRP3, ASC, and active caspase-1 p20 subunit under control, non-stimulated conditions; representative blots shown in **b**. ATP stimulation of LPS-primed cells alters expression of inflammasome components in cell lysates and extracellular media, quantified in **e** and **f** respectively. **g**: ATP stimulation of LPS-primed microglial cells leads to an increase in IL-1 β released into the extracellular media, an effect dose-dependently reduced by caspase-1 inhibitor Ac-YVAD-CMK. Data represented as mean \pm SEM. n=3-6 samples per group (**a – f**). Students t test, where *, ** p<0.05, 0.01 respectively when compared to unstimulated control cells. IL- β ELISA (**g**): n=3 experiments. From each experiment 3 samples per condition were analysed, and averaged to give overall data for each experiment. Data represented as mean of the 3 experiments \pm SEM. One-way ANOVA followed by Tukey's post-test, where *, ***, p<0.05, 0.001 respectively. Relevant comparison denoted by line on graph.

3.3. L5 spinal nerve transection causes behavioural hypersensitivity, spinal microgliosis, and macrophage infiltration of the DRG

Baseline mechanical and cold responses in adult male rats were assessed prior to any surgery. Following L5 spinal nerve transection (SNT) injury the change in behavioural sensitivities were assessed intermittently over a two week period, to ensure that the animals developed neuropathic pain-like behaviours. L5 SNT caused a significant decrease in hindpaw 50% withdrawal threshold to von Frey hairs, observed from the first time-point assessed; 3 days. This mechanical hypersensitivity lasted for the duration of the study, 14 days, and was only observed in the hindpaw ipsilateral to the injury, whereas contralateral thresholds were unaffected (Figure 1.3 a). Prior to L5 SNT injury, rats showed little to no response to the application of acetone to the plantar surface of the hindpaw. From as early as 3 days post-surgery the response to acetone applied to the ipsilateral hindpaw (licking, biting, and flicking) was significantly elevated. The response was strongest at 6 – 8 days post-surgery, and reduced again 10 days post-surgery (Figure 1.3 b).

Following L5 SNT injury a robust microgliosis was observed, visualised using IBA1, a marker for microglia and macrophages, immunohistochemistry. The number of IBA1-positive cells in the ipsilateral dorsal horn was significantly increased as early as 3 days post-surgery (4.90-fold increase \pm 0.95 compared to contralateral dorsal horn), was similarly elevated 7 days post-surgery (4.72-fold increase \pm 0.95, compared to contralateral dorsal horn), and was still significantly increased 21 days post-surgery (3.05-fold \pm 0.32, compared to contralateral dorsal horn), though numbers had begun to decrease from earlier timepoints. IBA1-positive cells were significantly increased when compared to both the contralateral dorsal horn, and also compared to the ipsilateral dorsal horn of 7-day sham animals. Interestingly, the number of IBA1-positive cells in the ipsilateral dorsal horn of sham animals was significantly greater than the number of IBA1-positive cells in the contralateral dorsal horn of the same animals (1.96-fold increase \pm 0.45), though still significantly less than numbers seen in L5 SNT animals at all timepoints examined (Figure 1.4 a and b). In addition to the increased number of microglia, it was noted that the morphology of the microglia after injury was altered; the cell bodies were

hypertrophied, and there were fewer processes extending from the cell body. The number of macrophages in the injured DRG was also examined following L5 SNT injury. The number of IBA1-positive cells was significantly higher than that in the contralateral L5 DRG, and in the L5 ipsilateral DRG of 7-day sham-injured animals from 3 days post-surgery (1.11-fold increase \pm 0.35, and 1.13-fold increase \pm 0.36, respectively. This number increased further 7- and 21 days post-surgery (respectively, 1.56-fold increase \pm 0.18 compared to contralateral, and 1.91-fold increase \pm 0.65 compared to contralateral). As with microglia in the spinal cord, the cell bodies of the macrophages in the injured DRG appeared hypertrophied when compared to the contralateral DRG. Cells appeared to form in a circular shape, surrounding large circular profiles presumed to be neurons which would be consistent with previous reports (Hu et al., 2007) (Figure 1.4 c – e).

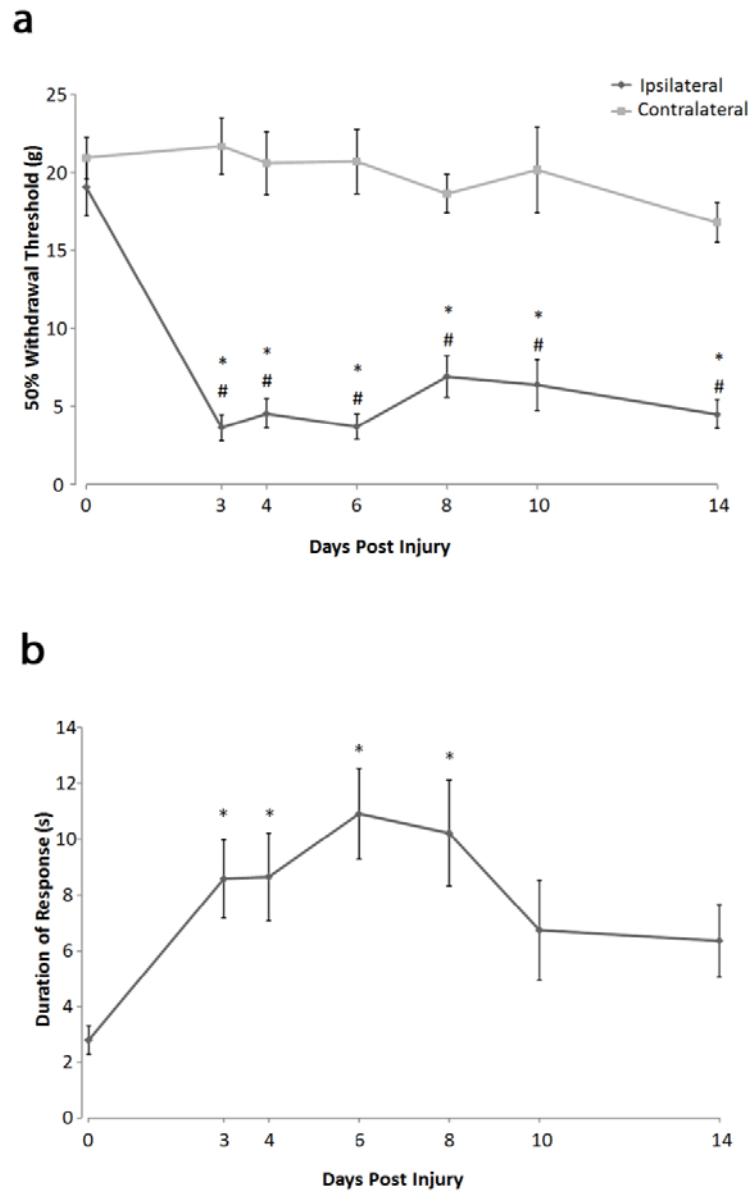


Figure 1.3: L5 spinal nerve transection causes lasting behavioural hypersensitivity

a: L5 SNT caused a significant decrease in hindpaw 50% withdrawal threshold to von Frey hairs, observed from the first time-point assessed, lasting up to two weeks. Contralateral hindpaw mechanical withdrawal thresholds were unaffected. **b:** Prior to injury, rats showed little to no response to the application of acetone to the plantar surface of the hindpaw. Following L5 SNT, duration of responses to acetone, licking, biting, and flicking of the hindpaw, were significantly elevated. Responses reduced and plateaued from 10 days post-surgery. Data represented as mean \pm SEM, $n=7$ animals. Two-way repeated measured ANOVA, followed by Dunnett's post-test, where *, #, $p<0.05$ compared to baseline responses, same-day contralateral responses (**a**). One-way repeated measures ANOVA, followed by Dunnett's post-test, where *, $p<0.05$ compared to baseline responses (**b**).

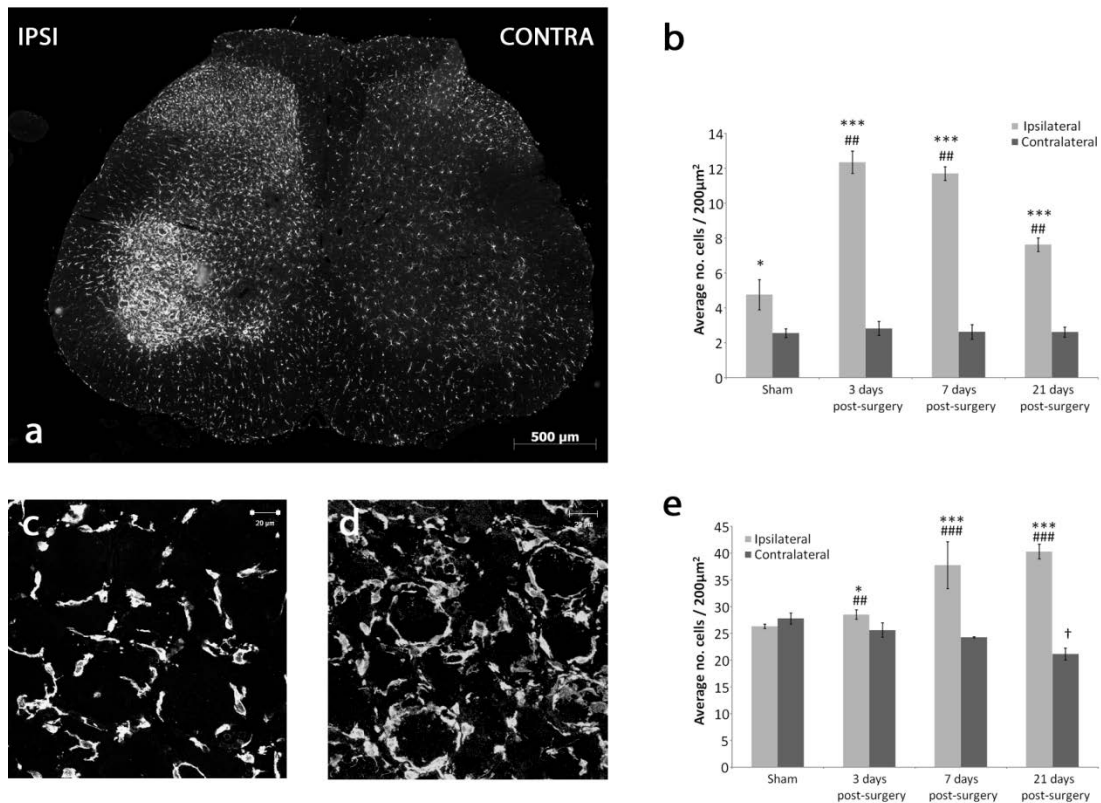


Figure 1.4: L5 spinal nerve transection causes spinal microgliosis and macrophage infiltration of the injured DRG

a: Lumbar spinal cord of an animal 7 days post-surgery, immunostained for microglia using IBA1 antibody. Scale 50 μ m. **b:** The number of cells in the ipsilateral superficial dorsal horn significantly increased from 3 days post-surgery, remaining elevated 21 days post-surgery. $n=3-5$ animals per timepoint, with at least 3 spinal cord sections analysed per animal. Analysis was carried out blind to timepoint. Two-way ANOVA with Dunnett's post-test, where *,*** $p<0.05$, 0.001 respectively compared to same day contralateral superficial dorsal horn, where ## $p<0.01$ compared to 7-day sham ipsilateral dorsal horn. **c and d:** L5 contralateral and ipsilateral DRG respectively of an animal 7 days post-surgery, immunostained for macrophages using IBA1 antibody. Scale 20 μ m. **e:** The number of cells in the ipsilateral L5 DRG significantly increased from 3 days post-surgery, remaining elevated 21 days post-surgery. $n=3$ animals per timepoint, with ≥ 3 DRG sections analysed per animal. Analysis was carried out blind to timepoint. Data represented as mean \pm SEM. Two-way ANOVA on ranks (data were not normally distributed) with Dunnett's post-test, where *, ***, $p<0.05$, 0.001 respectively compared to 7-day sham ipsilateral L5 DRG, where ##, ###, $p<0.01$, 0.001 respectively compared to same day contralateral L5 DRG, where † $p<0.05$ when compared to 7-day sham contralateral L5 DRG.

3.4. *The NLRP3 inflammasome is dysregulated after L5 SNT*

Quantitative PCR and Western blot techniques were employed to determine if the expression of inflammasome components is altered in the ipsilateral lumbar dorsal horn and injured DRG following L5 SNT. At the mRNA level, NLRP3 expression increased significantly 7 days post-surgery in the ipsilateral lumbar dorsal horn compared to both naïve animals, and sham animals 7 days post-surgery (3.04-fold \pm 0.41, $p < 0.001$, and 2.89-fold \pm 0.39, $p < 0.001$ respectively), whereas expression of caspase-1 mRNA was significantly increased 3 days post-surgery in the ipsilateral lumbar dorsal horn (3.11-fold \pm 0.45, $p < 0.001$, and 1.74-fold \pm 0.25, $p < 0.05$, compared to naïve and sham animals respectively) (Figure 1.5). Changes in expression of ASC mRNA were not tested owing to a lack of specificity of primers for this transcript. At the protein level, expression of ASC was significantly increased 7 days post-injury (1.61-fold \pm 0.14, $p < 0.05$ compared to naïve control) in the ipsilateral lumbar dorsal horn. Expression of the 20kDa subunit of active caspase-1 was also significantly increased 7 days post-injury (2.96-fold \pm 0.59, $p < 0.05$ compared to naïve control) in the ipsilateral lumbar dorsal horn (Figure 1.6).

In the injured DRG, expression of NLRP3 mRNA was found to be significantly upregulated 3, 7, and 21 days post-surgery compared to naïve animals (4.99-fold \pm 1.20, 6.25-fold \pm 1.21, 4.48-fold \pm 0.57, respectively, $p < 0.05$), while expression of caspase-1 mRNA was significantly upregulated 7 days post-surgery (4.49-fold \pm 0.95 compared to naïve animals, 3.24-fold \pm 0.68 compared to sham animals, $p < 0.05$) (Figure 1.7). At the protein level, expression of the adaptor protein ASC within the L5 DRG significantly increased 7 and 21 days following nerve injury (4.06-fold \pm 0.19, $p < 0.01$, and 3.66-fold \pm 0.84, $p < 0.05$ compared to naïve control, respectively). A significant increase in the expression of the 20kDa active form of caspase-1 in the L5 DRG was also observed 7 days post-injury (7.35-fold \pm 1.92, $p < 0.05$ compared to naïve control) (Figure 1.8).

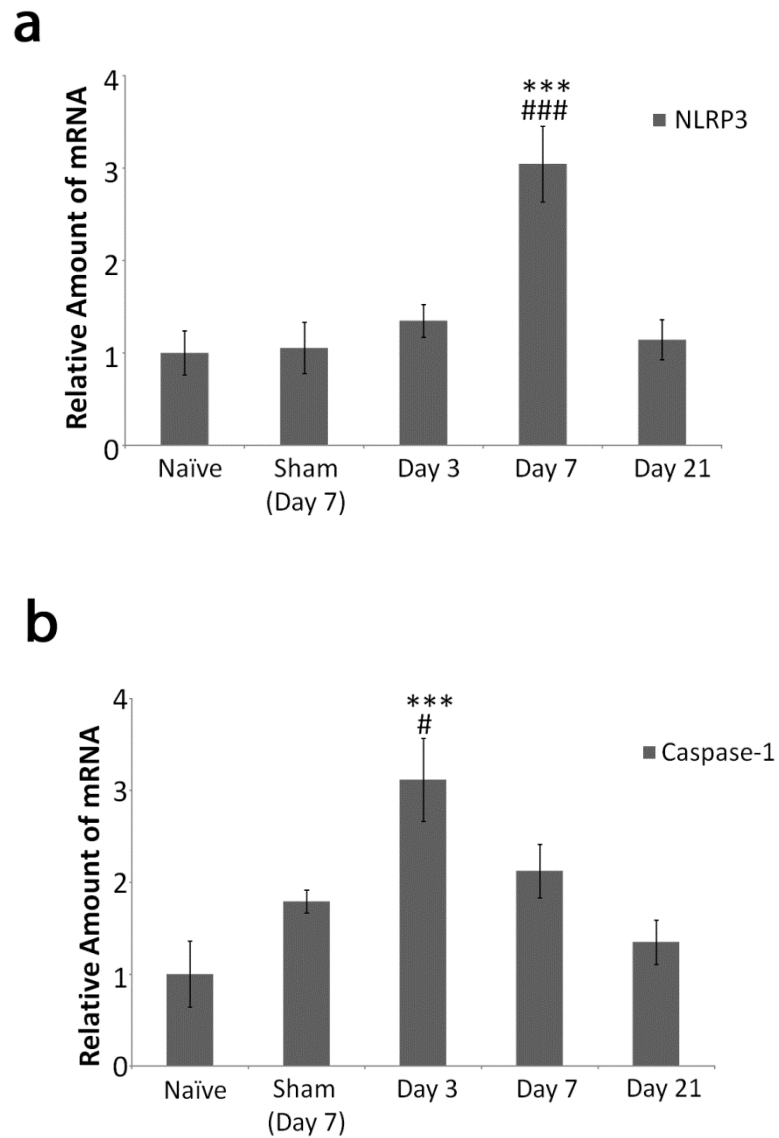


Figure 1.5: NLRP3 inflammasome components NLRP3 and caspase-1 mRNA is dysregulated in the ipsilateral lumbar dorsal horn after L5 SNT

Ipsilateral lumbar dorsal horn expression of NLRP3 **(a)** and caspase-1 **(b)** mRNA increases significantly following L5 SNT, as assessed by quantitative real-time PCR. One-way ANOVA with Dunnett's post-test, where ^{***},# $p < 0.001$, 0.05 compared to naïve animals and sham animals respectively. $n = 4-8$ animals per timepoint (1 ipsilateral lumbar dorsal horn per animal).

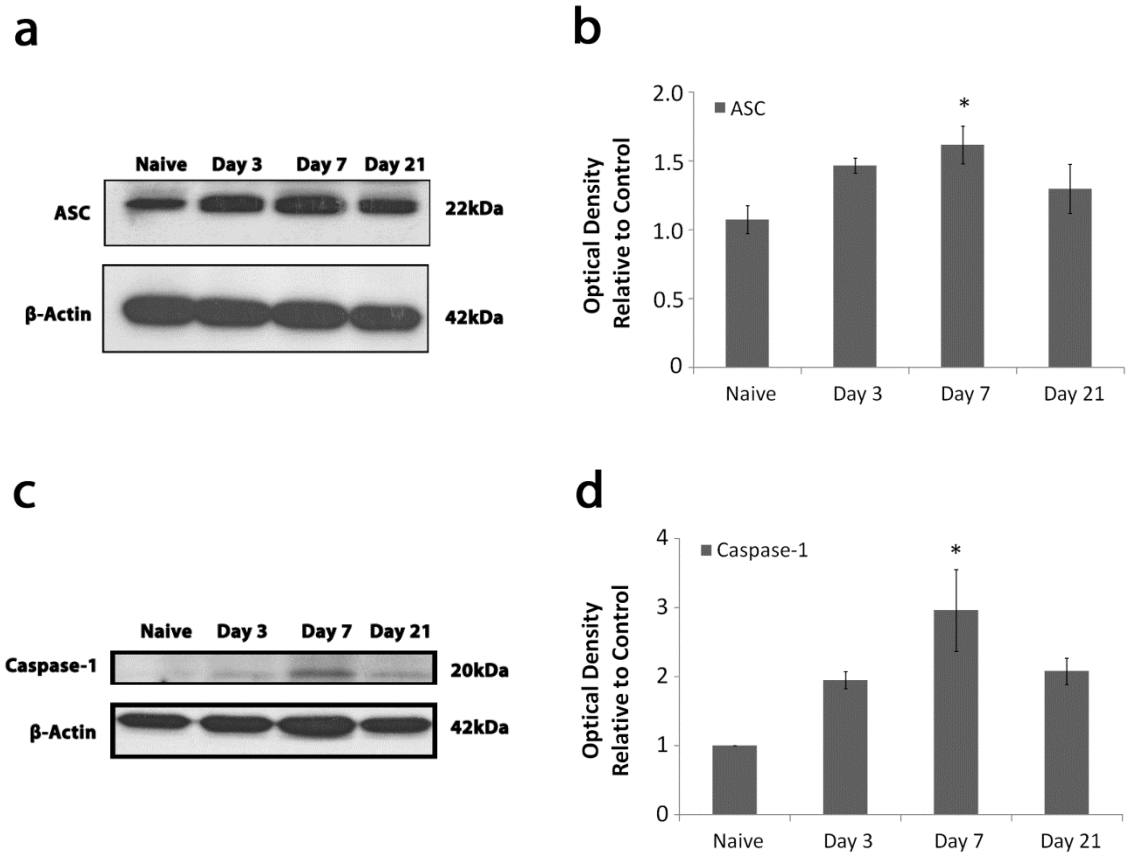


Figure 1.6: NLRP3 inflammasome components ASC and caspase-1 protein expression is dysregulated in the ipsilateral lumbar dorsal horn after L5 SNT

Ipsilateral lumbar dorsal horn expression of ASC **(a)** and caspase-1 **(c)** protein increases significantly following L5 SNT, quantified in **b** and **d**. One-way ANOVA with Dunnett's post-test (ASC), and one-way ANOVA on ranks with Dunn's post-test (caspase-1), where * $p < 0.05$ compared to naïve animals. $n = 3-4$ animals per timepoint (1 ipsilateral lumbar dorsal horn per animal).

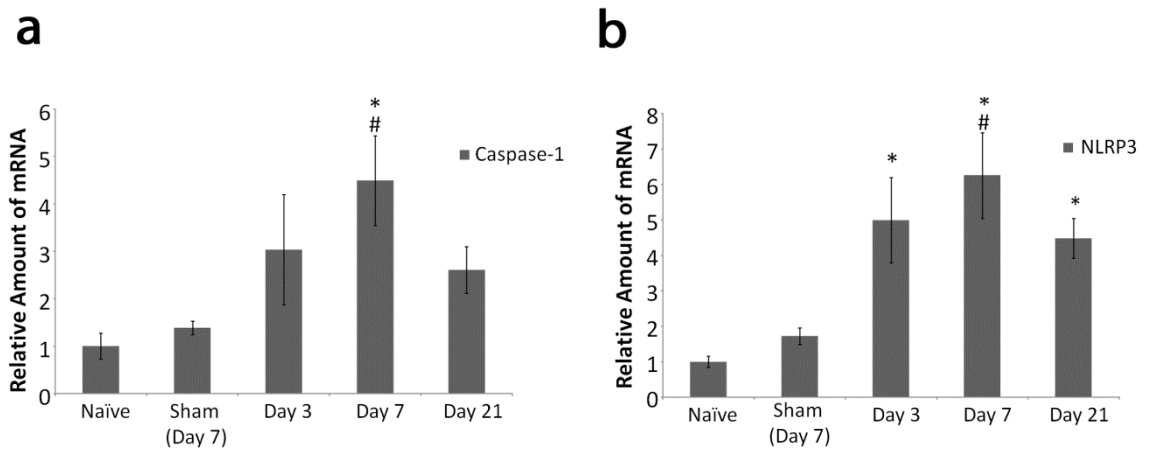


Figure 1.7: NLRP3 inflammasome components NLRP3 and caspase-1 mRNA is dysregulated in the ipsilateral L5 DRG after L5 SNT

Ipsilateral lumbar L5 DRG expression of NLRP3 **(a)** and caspase-1 **(b)** mRNA increases significantly following L5 SNT, as assessed by quantitative real-time PCR. One-way ANOVA on ranks (NLRP3) with Dunn's post-test, where *, # $p < 0.05$ compared to naïve animals and sham animals respectively. One-way ANOVA (caspase-1) with Dunnett's post-test, where *, # $p < 0.05$ compared to naïve animals and sham animals respectively. $n = 4-8$ animals per timepoint (1 whole DRG per animal).

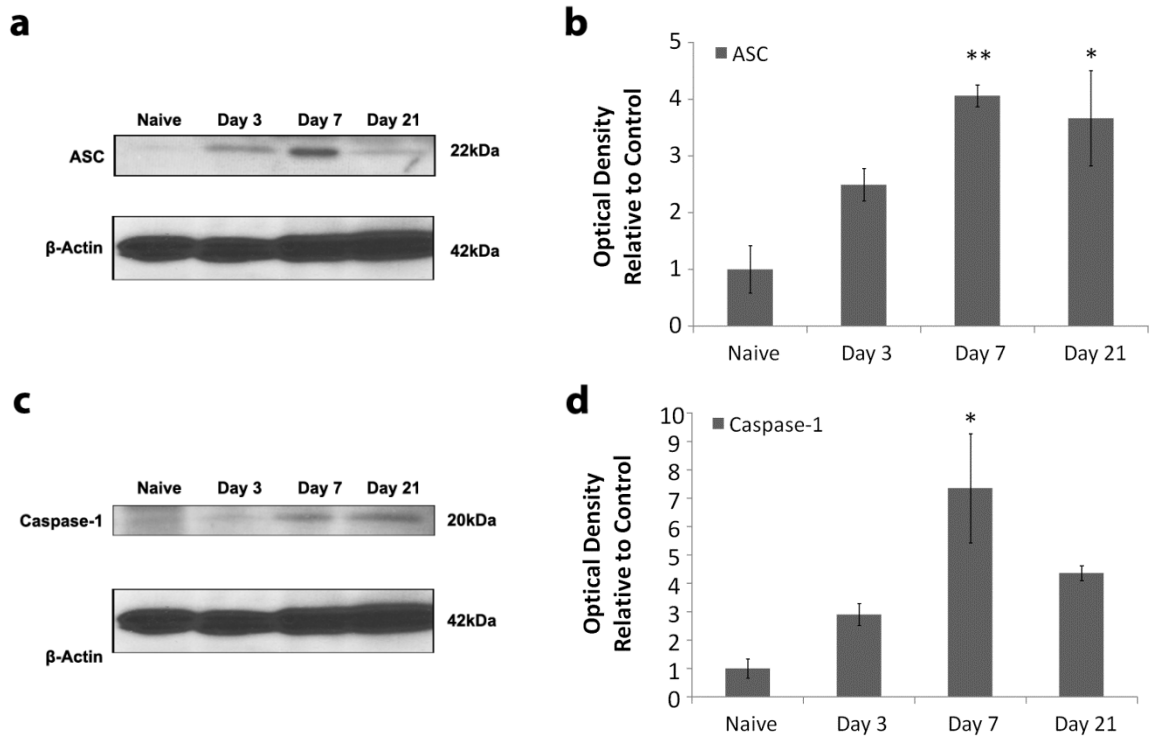


Figure 1.8: NLRP3 inflammasome components ASC and caspase-1 protein expression is dysregulated in the ipsilateral L5 DRG after L5 SNT

Ipsilateral L5 DRG expression of ASC (**a**) and caspase-1 (**c**) protein increases significantly following L5 SNT, quantified in **b** and **d**. One-way ANOVA with Dunnett's post-test where *,** $p < 0.05, 0.01$ compared to naïve animals. $n = 3-4$ animals per timepoint (1 whole DRG per animal).

3.5. *Dysregulation of inflammasome components occurs within microglia and macrophages*

Immunohistochemical analysis revealed expression of ASC within the lumbar spinal cord. ASC positive cells were found to have a ramified morphology, typical of microglia. To further investigate the origin of ASC expression, lumbar spinal cord sections were co-stained with either IBA1 (to identify microglia), GFAP (to identify astrocytes) or NeuN (to label neurons). Little to no co-localisation was observed with either GFAP or NeuN (<1%), whereas extensive co-localisation was observed with IBA1 (>97% ASC-positive cells expressing IBA1 at all timepoints examined (Figure 1.9). As discussed above, peripheral nerve injury leads to a marked increase in number and altered morphology (hypertrophy of the cell body and retraction of processes) of microglia in rodents; a process termed “microgliosis”. Following L5 SNT there was a marked increase in ASC immunoreactive profiles in superficial laminae of the ipsilateral dorsal horn, with a similar timecourse to that seen for IBA1-positive cells (see Figure 1.4). These ASC immunoreactive cells had the characteristic morphology of “effector” microglia. Increased numbers of ASC immunoreactive microglia were also observed around the soma of motor neurons within the ventral horn of the spinal cord.

Within the injured L5 DRG, ASC-positive cells were also found to co-localise extensively with IBA1 (<98% ASC-positive cells expressing IBA1 at all timepoints examined). As with IBA1 immunoreactive profiles, ASC-positive cells in the injured DRG took on a hypertrophied appearance compared to those seen in the contralateral DRG (Figure 1.10 a and b). Following L5 SNT there was a marked increase in ASC-positive cells, with a similar timecourse to that seen for IBA1-positive cells (Figure 1.10 c).

Finally, Immunostaining of the lumbar spinal cord for caspase-1 revealed an increase in caspase- positive cells in the superficial laminae of the dorsal horn following nerve injury. Similar to ASC, extensive co-localisation was observed with IBA1 in the superficial laminae (>85% caspase-1-positive cells expressing IBA1 at all timepoints examined), with little to no co-localisation with GFAP or NeuN. In the deeper laminae of the dorsal horn and the ventral horn

occasional neurons were weakly caspase-1 immunoreactive (Figure 1.11 a, and also refer to Appendix figure a.5). Similar to data obtained from IBA1 and ASC immunostaining, the number of caspase-1 immunoreactive profiles in the ipsilateral superficial lumbar dorsal horn increased significantly 3, 7, and 21 days-post surgery compared to both 7-day sham animals and the contralateral dorsal horn (see Figure 1.11 c).

These co-localisation studies suggest that the increased expression of the NLRP3 inflammasome components following L5 SNT is likely to be due to increased numbers of macrophages within the injured DRG and microglia within the lumbar spinal cord.

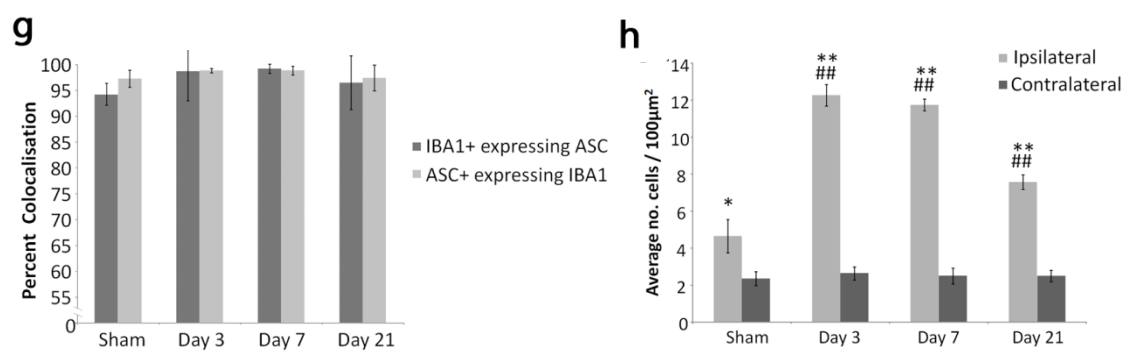
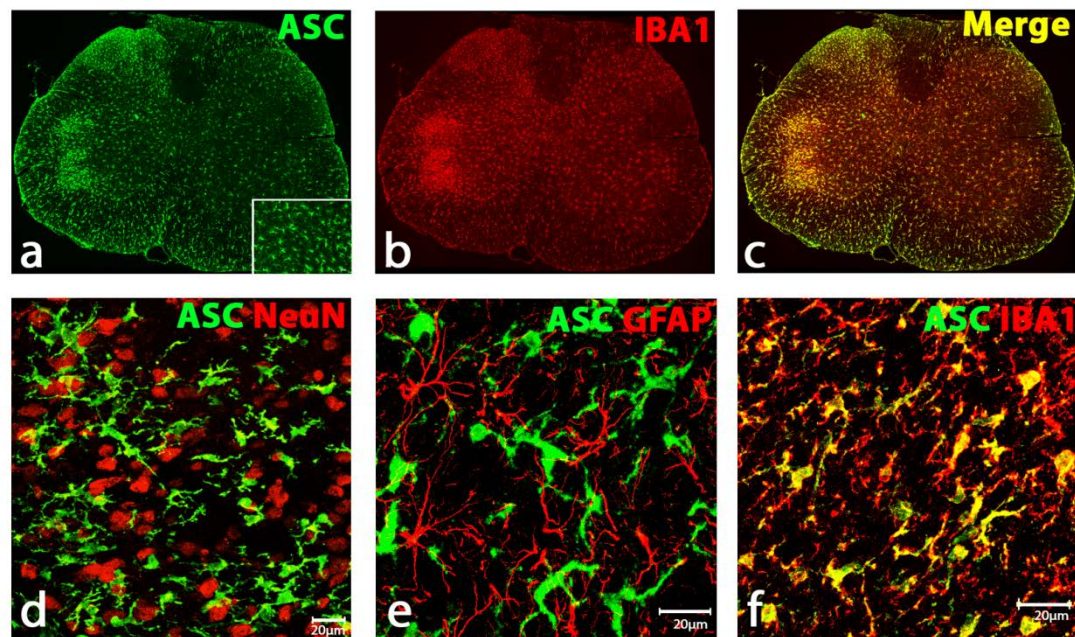


Figure 1.9: (figure legend on the next page)

Figure 1.9: Inflammasome adaptor ASC is highly expressed in microglia in the spinal cord

a: Immunohistochemical analysis reveals expression of ASC within the lumbar spinal cord. ASC positive cells were found to have a ramified morphology (inset), typical of microglia. **b and c:** Co-staining of lumbar spinal cord sections with IBA1 reveals a strong co-localisation, indicating this protein is expressed in microglia. ASC represented in the green channel, IBA1 represented in the red channel. Confocal microscopy reveals little to no co-localisation of ASC (green channel) with NeuN (red channel), **d**, or GFAP (red channel), **e**, but extensive co-localisation with IBA1 (red channel), **f**. Scale 20 μ m. **g:** ASC shows >95% co-localisation with IBA1 at all timepoints examined. **h:** The number of ASC-positive cells within the ipsilateral lumbar dorsal horn significantly increases from 3 days post-surgery, in a manner similar to that observed for IBA1-positive cells (see Figure 1.4). n=3-5 animals per timepoint, with ≥ 3 spinal cord sections analysed per animal. Two-way ANOVA with Dunnett's post-test, where *, ** $p < 0.05$, 0.01 respectively compared to same day contralateral superficial dorsal horn, where ## $p < 0.01$ compared to 7 day sham ipsilateral superficial dorsal horn.

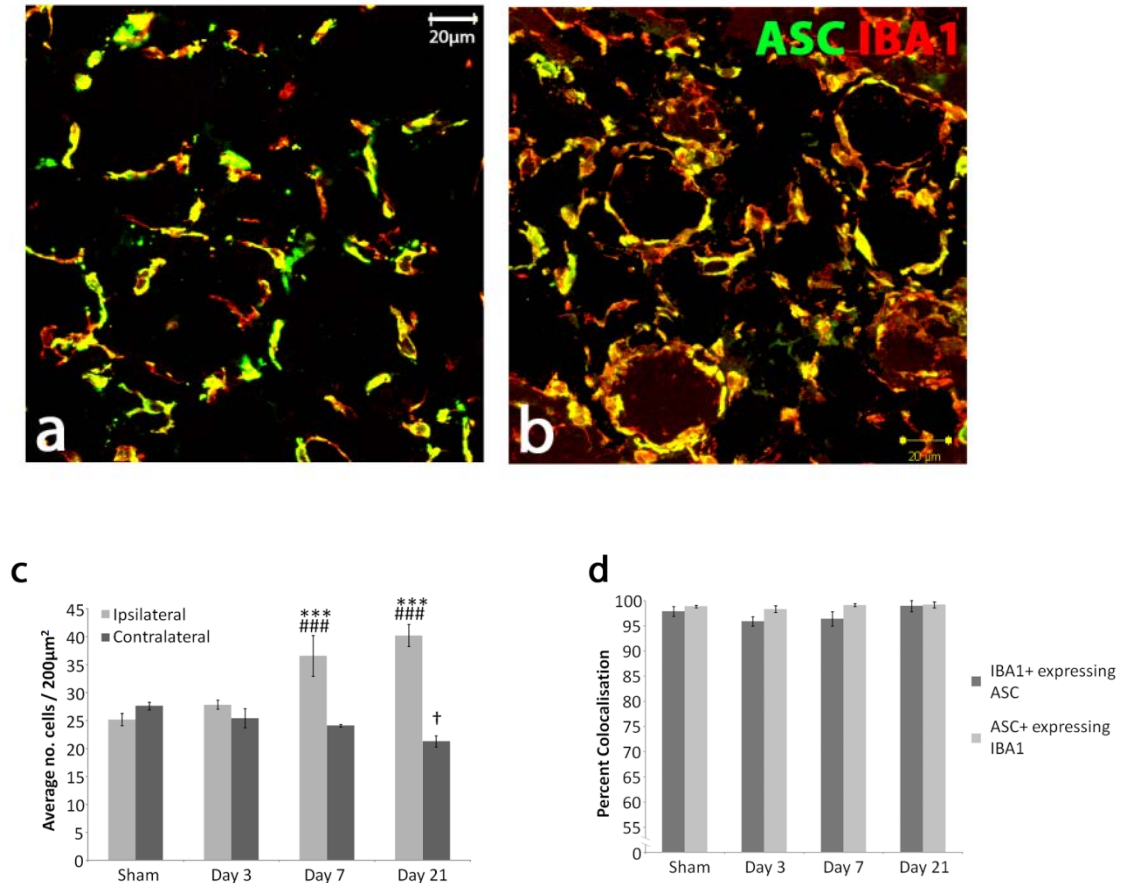


Figure 1.10: Inflammasome adaptor protein ASC is highly expressed in macrophages in the DRG

a: Immunohistochemical analysis reveals expression of ASC within the contralateral L5 DRG. Confocal microscopy reveals extensive co-localisation with IBA1. **b:** ASC is also extensively co-localised with IBA1 in the injured ipsilateral L5 DRG. Cells appear in a circular shape, surrounding circular profiles presumed to be neurons. ASC is represented in the green channel, and IBA1 is represented in the red channel. Scale 20 μm. **c:** ASC shows >95% co-localisation with IBA1 at all timepoints examined. **d:** The number of ASC-positive cells in the ipsilateral injured L5 DRG significantly increases from 3 days post-surgery, in a manner similar to that observed for IBA1-positive cells (see Figure 1.5). n=3-5 animals per timepoint, with ≥3 DRG sections analysed per animal. Two-way ANOVA with Dunnett's post-test, where *** p<0.001 compared to same day contralateral L5 DRG, where ### p<0.001 compared to 7 day sham ipsilateral L5 DRG, where † p<0.05 compared to 7 day sham contralateral L5 DRG.

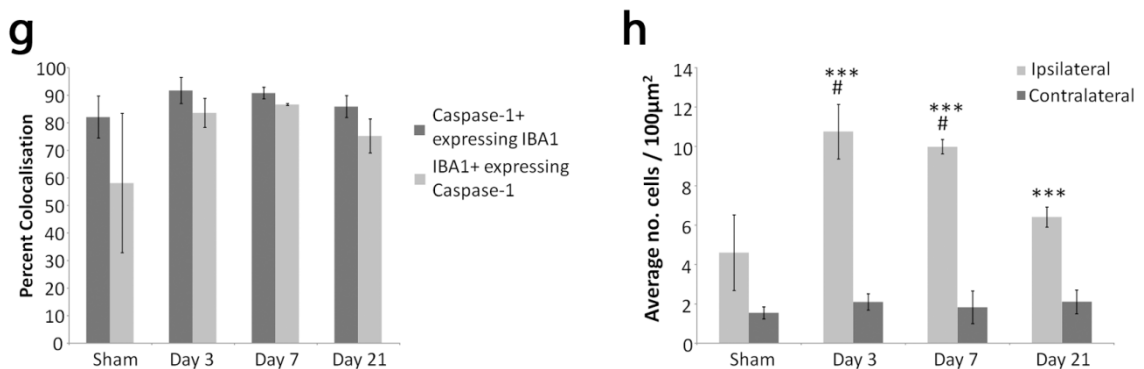
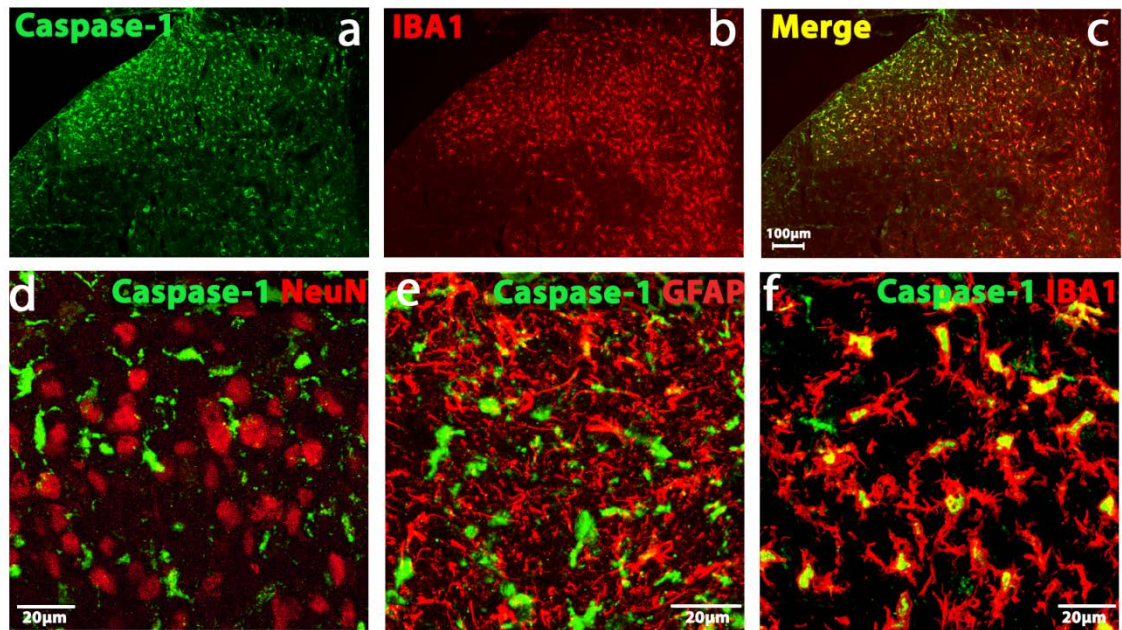


Figure 1.11: Caspase-1 is highly expressed in microglia in the spinal cord

a: Immunohistochemical analysis reveals expression of caspase-1 within the lumbar spinal cord. **b and c:** Co-staining of lumbar spinal cord sections with IBA1 reveals a strong co-localisation, indicating this protein is expressed in microglia. Scale 100 µm. Confocal microscopy in the superficial dorsal horn reveals little to no co-localisation with NeuN, **d**, or GFAP, **e**, but extensive co-localisation with IBA1, **f**. Scale 20 µm. **g:** Caspase-1 shows high co-localisation with IBA1 at all timepoints examined. **h:** The number of Caspase-1-positive cells significantly increases from 3 days post-surgery, in a manner similar to that observed for IBA1-positive cells (see Figure 1.5). Data represented as mean \pm SEM, $n=3-5$ animals per timepoint, with ≥ 3 spinal cord sections analysed per animal. Two-way ANOVA with Dunnett's post-test, where *** $p < 0.001$ compared to same day contralateral superficial dorsal horn, where # $p < 0.05$ compared to 7 day sham ipsilateral superficial dorsal horn. Caspase-1 antibody detects both pro-caspase-1 and active caspase-1.

4. Discussion

Several studies have previously shown a role for IL-1 β in pain states. For example injection of IL-1 β into a variety of tissues elicits a potent hyperalgesic response while in various models of inflammatory and neuropathic pain IL-1 β expression has been shown to increase in the injured nerve, DRG, and spinal cord (Ren and Torres, 2009). Release of IL-1 β is a complex, multi-step process, requiring first assembly of the inflammasome in order to effect activation of caspase-1, the enzyme responsible for the cleavage of pro-IL-1 β into the mature, secreted, cytokine. Uncontrolled and abnormal inflammasome activation, leading to aberrant release of IL-1 β has been demonstrated in autoinflammatory diseases such as the CAPS syndromes in which there is a mutation in the cryopyrin gene. Symptoms of these disorders include rash, fever/chills, and joint and muscle pain (Petrilli and Martinon, 2007). Following peripheral nerve injury in rodents there is a robust microgliosis in the spinal cord, as well as infiltration of the DRG by macrophages, a key feature of which is the release of pro-inflammatory cytokines including TNF- α and IL-1 β (McMahon and Malcangio, 2009, Calvo et al., 2012). Thus we explored whether the inflammasome, responsible for regulating the cleavage and release of inflammatory cytokines IL-1 β and IL-18, which in turn act to increase the transcription of other pro-inflammatory cytokines, plays a role in neuropathic pain.

There are few publications in which the inflammasome has been investigated in microglia. In 2008, Halle and colleagues carried out work looking at the response of primary microglia to Amyloid β (A β) (Halle et al., 2008). It was shown that microglia release IL-1 β in response to incubation with A β , resulting in frustrated phagocytosis and Cathepsin B release from ruptured lysosomes, a protease that has been implicated in NLRP3 inflammasome activation (Hornung et al., 2008). However, the publication did not directly show the presence of inflammasome components in this cell type. More recently work has been published which shows a reduction in *S. aureus*-induced IL-1 β , though interestingly not IL-18, release from primary microglia cultured from ASC and NLRP3 knockout mice compared to wildtype littermates, suggesting

that microglia do express the NLRP3 inflammasome (Hanamsagar et al., 2011). Early work by Kingham and colleagues had showed caspase-1 activation in microglia activated with chromogranin A. Interestingly the apoptotic effects mediated by caspase-1 activation were found to not directly involve the production of IL-1 β (Kingham et al., 1999, Kingham and Pocock, 2000).

Data presented here show that the NLRP3 inflammasome component proteins NLRP3, ASC, and caspase-1 are all expressed in primary cultured peritoneal macrophages, in line with previous reports, and also in primary cultured microglia, as determined by Western blot analysis. Cells were either primed with LPS for 4 hours, followed by a brief 15 minute stimulation with high concentration ATP, or treated with vehicle and so essentially unstimulated. This experimental paradigm was chosen in line with that published by other groups using LPS/ATP stimulation of macrophages to study inflammasome activation (Sanz and Di Virgilio, 2000, Mariathasan et al., 2004, Qu et al., 2007). It is known that inflammasome expression must be induced *in vitro*, and LPS stimulation is known to license NLRP3 upregulation via NF- κ B activation (Bauernfeind et al., 2009). There are several known activators of the NLRP3 inflammasome, as discussed in the General Introduction. The choice of ATP as an activator in these experiments was in line with previously published reports investigating inflammasome activation in macrophages which were used as a positive control in these experiments. Additionally it is thought that ATP released from damaged neurons contributes to microglial activation and subsequent development of behavioural hypersensitivity in animal models of peripheral nerve injury (Tsuda et al., 2003, Coull et al., 2005, Clark et al., 2010b, Beggs et al., 2012, Trang et al., 2012).

The overall pattern of inflammasome protein expression and release following stimulation was very similar in microglial cells to that seen in macrophages. In control, unstimulated, cells there was little to no expression of NLRP3. Expression of this protein was rapidly upregulated following LPS/ATP stimulation, however. It is widely reported that NLRP3 expression in macrophages is inducible upon exposure to inflammatory stimuli (O'Connor et

al., 2003, Bauernfeind et al., 2009, Franchi et al., 2009), and this data is also consistent with the report that circulating macrophages from naïve animals have only a modest level of NLRP3 expression (Guarda et al., 2011). This study shows that NLRP3 expression is similarly inducible in microglia, with little to no constitutive expression. The adaptor protein ASC was present in the cell lysates of unstimulated macrophages and microglia. Following cell stimulation, the amount of ASC detected in cell lysates decreased with a concomitant increase detected in the extracellular media. This is in agreement with published reports showing that, along with mature IL-1 β and active caspase-1, ASC is quickly ejected from the cell following stimulation both *in vitro* and *ex vivo* (Mariathasan et al., 2004, Clark et al., 2006, Qu et al., 2007). That there was some ASC protein detected in the extracellular media of unstimulated cells was unexpected. This may reflect some constitutive release of the protein from cells, possibly as a regulatory mechanism aimed at controlling the amount of IL-1 β activation or release. Alternatively this may reflect the fact that, by necessity for the purposes of gaining enough protein for detection, cells were densely plated. This may have resulted in a low level of activation. However, active caspase-1 was not detected in the extracellular media, arguing against this possibility.

There was a small amount of active caspase-1 present in the lysates of unstimulated cells. This was significantly increased following LPS/ATP stimulation. At the same time, active caspase-1 was detected in the extracellular media of stimulated cells, whilst it was undetectable in the extracellular media of control, unstimulated, cells. ATP stimulation of LPS-primed microglia resulted in IL-1 β release into the extracellular media, an endpoint in the inflammasome activation pathway. Pre-treatment with the cell-permeant inhibitor of the enzymatic activity of caspase-1, Ac-YVAD-CMK, dose-dependently decreased the amount of IL-1 β released from stimulated cells. IL-1 β release was not completely abolished. This may either be due to an insufficient concentration of inhibitor used, or alternatively may indicate that other, caspase-1-independent, pathways leading to IL-1 β release may be present in these cells. It was previously known that brief stimulation of LPS-primed microglia with ATP resulted in the release of IL-1 β (Sanz and Di Virgilio,

2000, Clark et al., 2006, Calvo et al., 2010). This data indicates that this release is, at least in part, mediated by the activation of the NLRP3 inflammasome and activation of caspase-1.

It should be noted that while the release of ASC, caspase-1, and IL-1 β is postulated to have come about as a result of inflammasome activation, the possibility that the presence of these proteins in the extracellular media occurred as a result of cell death cannot be excluded. Following cell stimulation, media were collected and immediately frozen. Any cells which had detached from the well's surface, which may occur following cell death, would have remained in the media and ruptured upon freezing. This represents a flaw in the protocol. If the experiments were to be repeated in the future, the media should be centrifuged, and any debris discarded. This would remove the potential confounds of proteins released from ruptured cells, and increase certainty in the subsequent findings.

L5 SNT is a commonly used animal model of neuropathic pain, which results in robust behavioural hypersensitivity as well as a robust microgliosis of the lumbar spinal cord and increased numbers of macrophage within the DRG. In this study, L5 SNT resulted in long-lasting mechanical hypersensitivity in rats as well as a hypersensitivity to cold stimulus, though this was not as long-lasting. L5 SNT resulted in a microgliosis of the lumbar spinal cord, in a timecourse similar to that reported previously (Scholz et al., 2008), as well as increased numbers of macrophages within the DRG similar to published reports (Hu and McLachlan, 2002). It is not possible to tell from these experiments if these numbers represent migration of cells, proliferation, or both.

Having established that the inflammasome is expressed in both macrophages and microglia *in vitro*, we next sought to determine if inflammasome expression was altered in an animal model of neuropathic pain, given the importance of macrophages and microglia in the development of pain-like behaviours in such models. Using a combination of qPCR and Western blot analysis it was determined that inflammasome components NLRP3, ASC, and caspase-1 are

all dysregulated in both the spinal cord and DRG following L5 SNT in rats. All proteins showed a similar pattern of dysregulation, with increases beginning 3 days after injury, generally maximal after 7 days, but remaining elevated to some extent 21 days after injury. This data is confirmed in independent experiments carried out by Dr. Ana Antunes-Martins, using next generation genome sequencing techniques, which revealed a significant increase in NLRP3, Pycard (ASC), and caspase-1 cDNA in rat ipsilateral lumbar dorsal horns and L5 DRG 7 days after L5 SNT injury (see Appendix).

Further investigation using immunohistochemical analysis revealed that ASC-positive cells in the lumbar spinal cord had a ramified morphology, typical of that of microglia. Co-staining with IBA1 revealed that ASC was almost exclusively expressed by microglia in the spinal cord in both sham-operated animals as well as SNT-injured animals at all timepoints examined. ASC was not seen to be present in either astrocytes or neurons. This is in disagreement with a previous report stating that ASC was found to be present in astrocytes, oligodendrocytes, and neurons, as well as in microglia in the spinal cord (de Rivero Vaccari et al., 2008). The reason for this discrepancy is unclear; although the group did use a different antibody. The antibody used in this study is specific to ASC since ASC-positive staining disappears in tissues obtained from ASC^{-/-} mice (see Chapter 3). Additionally it was reported that following spinal cord injury ASC was upregulated in motor neurons in the ventral horn of the spinal cord. This was not observed in this study; though the location and severity of injury differed between studies and differences in the changes in expression profiles of different cell types would be expected.

In the present study ASC immunofluorescence appeared to be present throughout the cells, including in the smaller branched processes of cells in a more quiescent state, usually observed contralateral to the injury. This differs from reports of cellular distribution of ASC *in vitro* where activation of the inflammasome or pyroptosome results in ASC immunostaining taking on a speck-like appearance, from which it gains its name (Masumoto et al., 1999, Yu et al., 2006, Fernandes-Alnemri et al., 2007, Halle et al., 2008). Potentially this indicates that inflammasome or pyroptosome formation did not occur

following L5 SNL. Alternatively it could indicate that the protein behaves differently in artificial *in vitro* conditions. Indeed it has been suggested that the formation of these characteristic specks occurs as a result of forced overexpression of ASC in these cell systems (Bryan et al., 2009). Immunostaining for ASC was also carried out in the DRG. As in the spinal cord, there was extensive colocalisation with IBA1 (>95% at all time points examined), indicating it was present almost exclusively in macrophages in this tissue.

Immunostaining for caspase-1 in the lumbar spinal cord also showed extensive co-localisation with microglia. However the staining differed from that of ASC in that caspase-1 staining was less extensively localised within the microglial processes. Additionally co-localisation was not as extensive as that of ASC. It is not possible to determine the state of activation of caspase-1 in microglia in this experiment as the antibody used detects both the p45 subunit of pro-caspase-1 and the p20 subunit of the cleaved active caspase-1. In this study, caspase-1 staining appeared to be localised to the nucleus, and rarely visualised in microglial processes. This raises the possibility that the staining observed was non-specific and instead an artefact of overexposure. However examination of confocal microscopy images of caspase-1 and DAPI co-localisation suggest this is not the case (refer to Appendix, figure a.5).

That caspase-1 co-localised with microglial marker IBA1 in this study is in agreement with the observations of Kawasaki *et al.* who reported some, but not complete, co-localisation with microglial marker IBA1 in the lumbar dorsal horn of SNL-injured rats (Kawasaki et al., 2008a). However the group also reported no injury-induced change in the level of caspase-1 protein levels in the dorsal horn of SNL-injured rats at either 1- or 10- days post-injury, which is in disagreement with data presented here. A significant increase in active caspase-1 protein levels in the ipsilateral lumbar dorsal horn was observed only 7 days post-surgery in this study. Potentially this increase is transient, and protein levels may be reduced again to non-significant levels by 10 days post-surgery. The group also reported no change in caspase-1 protein expression in the injured DRG 1 day after injury. As with the ipsilateral lumbar dorsal horn, a

significant change in active caspase-1 protein level in the injured DRG was not detected until 7 days after injury. Thus it is possible that the group did not detect a change owing to the early time point investigated.

Data presented here show that, at least in the L5 SNT model of neuropathic pain, the inflammasome is dysregulated after peripheral nerve injury. Several lines of evidence indicate that this dysregulation occurs in macrophages within the DRG, and microglia in the spinal cord. Firstly the temporal pattern of dysregulation of both mRNA message and protein expression is similar to that of the macrophage infiltration observed in the DRG and microgliosis observed in the spinal cord in this study, and others. Data presented here show that the inflammasome components NLRP3, ASC, and caspase-1 are all expressed *in vitro* in primary macrophages and microglia. This was previously shown for macrophages (Sanz and Di Virgilio, 2000, Mariathasan et al., 2004, Qu et al., 2007), and the presence of caspase-1 in microglia had been previously reported (Kingham et al., 1999, Kingham and Pocock, 2000). Finally ASC and caspase-1 immunopositive cells were found to co-localise extensively with microglial marker IBA1 within the spinal cord. The increase in inflammasome message and protein expression may reflect the infiltration of macrophages and microglia in the DRG and spinal cord respectively. However, given that stimulation of macrophages and microglia *in vitro* led to an increase in inflammasome component expression either in the cell lysates or within the cell media, it is also possible that within the macrophages and microglia themselves, likely in an “effector” state, there is also some increase in inflammasome expression contributing to the observed increases.

Having established that L5 SNT leads to increases in inflammasome components, it will be of interest to determine if this contributes to the behavioural hypersensitivity associated with L5 SNT injury. Further experiments will focus on the effect of inhibition of the inflammasome at different levels of the pathway on both peripheral nerve injury-induced behavioural hypersensitivity as well as the associated microglial changes in the spinal cord.

Chapter 2: Inhibition of Caspase-1 Activity Attenuates Behavioural Hypersensitivity in L5 SNT Model of Neuropathic Pain, and LPS-Induced Hindpaw Hypersensitivity

1. Introduction

In the previous chapter, it was shown that L5 SNT injury in rats causes dysregulation of the NLRP3 inflammasome, with ASC found to be expressed in both macrophages and microglia in the DRG and lumbar spinal cord respectively, and caspase-1 expressed in microglia in the spinal cord. However, it was unclear as to whether this change merely reflected a change in cell numbers, given that both macrophage and microglia cell numbers were shown to increase in the injured DRG and ipsilateral lumbar dorsal horn, or if inflammasome protein expression within these cells was concomitantly increased. Subsequent work aims to disrupt inflammasome signalling in order to investigate whether or not inflammasome assembly and activation plays a functional role in animal models of persistent pain, and associated microgliosis.

Cleavage of pro-IL-1 β into the mature cytokine is the endpoint of the inflammasome pathway. IL-1 β is known to contribute to the generation of pain states in various animal models of pain, and injection of IL-1 β into various tissues generates behavioural hypersensitivity in rodents (Ren and Torres, 2009). In this chapter the focus is on the inhibition of caspase-1, upstream of IL-1 β activation. Caspase-1, originally called interleukin converting enzyme, or ICE, was first described following attempts to purify the enzyme responsible for the cleavage of pro-IL-1 β (Thornberry et al., 1992). Pro-caspase-1 is recruited to the inflammasome complex via an N-terminal CARD domain (Lamkanfi and Dixit, 2009). Pro-caspase-1 is an autolytic enzyme meaning that it is able to enzymatically cleave, and subsequently activate, other pro-caspase-1 molecules when in close proximity following recruitment to the inflammasome complex. Cleavage of the larger 45kDa pro-caspase-1 molecule results in dissociation of the active heterodimer complex caspase-1 molecule which

consists of two p10 and two 20 subunits (Boatright et al., 2003, Boatright and Salvesen, 2003, Martinon and Tschopp, 2004).

Experiments presented in this chapter aimed to disrupt inflammasome signalling in order to investigate whether or not inflammasome assembly and activation plays a functional role in animal models of persistent pain, and associated microgliosis. Ac-YVAD-CMK is a cell-permeable irreversible peptide inhibitor of caspase-1 activity (Rabuffetti et al., 2000), and was used to determine if caspase-1 inhibition results in an alteration of behavioural hypersensitivity following L5 SNT, as an animal model of neuropathic pain, and in an animal model of central inflammatory pain.

2. Methods

2.1. *Animals and surgery*

Adult male Wistar rats (Harlan, UK) were used in accordance with UK Home Office regulations (Animals Scientific Procedures Act 1986).

L5 SNT: Animals received L5 SNT injury using the same method as described in Chapter 1.

Drugs and delivery: Ac-YVAD-CMK (Calbiochem) was administered as 20 nmol dissolved in 20 μ l 10% DMSO in sterile saline. Under isoflurane anaesthesia a 29G insulin syringe (Myjector U-100 Terumo) was inserted between the L5 and L6 vertebrae, where the spinal cord consists mainly of spinal roots. A volume of 20 μ l was injected, after which the needle was slowly removed. The accuracy of each injection was ensured through the observation of an injection-induced tail-flick (Mestre et al., 1994). Ac-YVAD-CMK or vehicle were administered intrathecally immediately before L5 SNT surgery, and thereafter daily in the first study. In the second study Ac-YVAD-CMK or vehicle was administered 5, 6, 7, and 8 days after surgery.

LPS-induced mechanical hypersensitivity: Under isoflurane anaesthesia rats were intrathecally injected into the rat lumbar region, between the L5 and L6 vertebrae using a 29 G 13mm needle, with LPS (2 μ g dissolved in 10 μ l of 0.9% sterile saline) twice; the first dose, to prime the immune system, the second was administered 24 hours later, concomitant with either vehicle (10% DMSO in 0.9% sterile saline), or 20 nmol Ac-YVAD-CMK (Calbiochem). Mechanical withdrawal thresholds were assessed 30 minutes, 1 hour, and 3 hours after the second injection of LPS. Animals were then sacrificed, and tissue collected.

As described in Chapter 1, animals were either sacrificed by terminal anaesthetisation and transcardially perfused with either 4% paraformaldehyde plus 1.5% picric acid for histology, or saline alone for Western blot analysis. Tissue used for histology: Lumbar spinal cords were excised and post-fixed overnight, dorsal root ganglia (DRG) were post-fixed for 3-4 hours. Tissue was cryoprotected in 20% sucrose in 0.1M Phosphate Buffer (PB) for 4-7 days before OCT embedding. Tissue used for Western blot analysis: Lumbar spinal

cords were excised and quadrisectioned. Lumbar spinal cords and DRG were snap-frozen in liquid nitrogen and stored at -80°C.

2.2. Behavioural testing

Animals were tested in a temperature stable room during the light period of their day/night cycle, at the same time every day. Animals were placed in a polymethyl methacrylate box with a wire grid bottom and allowed to habituate for a period of 15-30 minutes before testing. Animals were trained once for each behavioural test, and then two baseline tests were performed on different days.

Mechanical testing: Mechanical withdrawal thresholds were assessed using calibrated von Frey hairs (Ugo Basile) according to the “up-down” method (Chaplan et al., 1994), as described in Chapter 1. The 50% withdrawal threshold was then calculated using the method described by Dixon (Dixon, 1980).

Cold testing: For measurement of cold hypersensitivity, a drop of acetone was applied to the plantar surface of the rat hindpaw, as described in Chapter 1. 3 tests were carried out, at least 10 minutes apart from each other, and an average response was recorded.

All behavioural testing was carried out blind to treatment.

2.3. Western blot

Tissues were homogenised in the same way as described in Chapter 1. Protein concentration of all samples was determined using a BCA Protein Assay kit (Thermo Scientific). Lysates were normalised to 3 5µg protein per sample. Proteins (35 µg lysate per well) were separated using SDS-PAGE (15% gel to visualise IL-1β) and transferred to 0.2 µm pore size PVDF membranes (Millipore). Membranes were blocked in 10% skimmed milk in PBS-T for 45 minutes before overnight incubation with primary antibody at 4°C as already described in Chapter 1. Species appropriate HRP-conjugated secondary antibody (1:10,000, Amersham) was applied the subsequent day for 1 hour at room temperature. Membranes were visualised using ECL-prime reagent for 5 min (GE Healthcare) before detection by autoradiography. For

visualisation of protein loading control, the membranes were stripped using a β -mecaptoethanol buffer as described in Chapter 1, before incubation with primary antibody.

Antibodies used: Rabbit anti-IL-1 β (1:800, Millipore), mouse anti- β -actin (1:15,000, Sigma), donkey anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:20,000, GE Healthcare), donkey anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:20,000, GE Healthcare).

Blots were analysed by determining mean intensity of each band using a fixed box size for all. The background intensity for each blot was then subtracted from all values obtained. All quantifications were then normalised against β -actin loading control. All blots were then compared to the mean band intensity for control-treated samples or naive animals. Analysis was carried out using Quantity One 1-D Analysis Software version 4.6.9 (Biorad).

2.4. Histology

Lumbar spinal cords were cryostat cut in 20 μ m sections and thaw-mounted onto glass slides. When slides were fully dry (at least 1 hour after tissue mounting), they were stored in cryoprotectant (30% ethylene glycol, 30% glycerol, 40% PBS) at -20°C until required.

Immunohistochemistry was carried out according to the methods described in Chapter 1. Primary antibodies used: Rabbit anti-GFAP (1:1000, DAKO), rabbit anti-IBA1 (1:1000, WAKO), rabbit anti-p-p38 (1:100, Cell Signalling). Tyramide signal amplification was used to visualise p-p38 (refer to Chapter 1 Methods, **Section 2.8**). Secondary antibodies: anti-mouse or anti-rabbit Alexa Fluor 488 (1:1000, Stratech), anti-mouse or anti-rabbit Alexa Fluor 546 (1:1000, Stratech), ExtrAvidin FITC (1:200, Sigma). Specificity of staining was determined by omission of the appropriate primary antibody as a negative control. Slides were mounted on coverslips using Vectashield mounting

medium with DAPI (Vectorlabs) and visualised using a Zeiss Axioplan 2 fluorescent microscope.

Quantification:

Cell counts / colocalisation: 3 100 μm^2 boxes were drawn over the superficial L5 dorsal horn, ipsilateral and contralateral to the injury. Cells which were associated with a nuclear stain, within the confines of the box, were counted. Counting was performed manually. An average count from the 3 boxes was taken. At least 3 sections per animal were analysed.

GFAP intensity: All GFAP images had been taken on the same day, using the same exposure. As for the cell counts, 3 100 μm^2 boxes were drawn over the superficial L5 dorsal horn, ipsilateral and contralateral to the injury. A 20 μm^2 box was drawn in the white matter, and was used to generate a background intensity value. The intensity of the 3 boxes, minus the background intensity value, was averaged. At least 3 sections per animal were analysed. All analysis was carried out blind to treatment. Analysis was carried out using AxioVision LE version 4 software.

2.5. Statistical analysis

All data are represented as mean \pm SEM where appropriate. Western blot, and histology data were analysed using one-way ANOVA followed by Dunnett's post-test. Behavioural data were analysed using two-way repeated measures ANOVA followed by Dunnett's post-test. Where data were not normally distributed, ANOVA tests were performed on ranked data. All analysis was carried out using the statistical package SigmaPlot for Windows, version 12.

3. Results

3.1. Pre-treatment with a caspase-1 inhibitor attenuates L5 SNT-induced behavioural hypersensitivity

To investigate the role of the inflammasome in SNT-induced pain-like behaviours, animals received intrathecal Ac-YVAD-CMK (20 nmol), a cell-permeant irreversible peptide inhibitor of caspase-1, or vehicle (10% DMSO) immediately before L5 SNT surgery, and thereafter daily injections to the end of the study. It was felt that this technique was sufficient for drug delivery to the lumbar dorsal horn since it has been shown that volumes as low as 5 μ l injected intrathecally reach the L3-L4 regions of the spinal cord (Xu et al., 2006). Rats were assessed for mechanical hypersensitivity 3, 5, and 7 days post-surgery. On days 5 and 7, rats treated with the caspase-1 inhibitor showed a significant increase in 50% paw withdrawal threshold compared to vehicle-treated animals ($p < 0.05$ on both days) (Figure 2.1 a). Cold hypersensitivity was assessed 2, 4, and 6 days post-surgery. On days 4 and 6 drug-treated animals had significantly decreased duration of responses to acetone compared to vehicle-treated animals ($p < 0.05$, and $p < 0.001$ respectively) (Figure 2.1 b).

Western blot of the injured DRG revealed a significant decrease in expression of both the pro-and mature form of IL-1 β in YVAD-treated animals compared to vehicle (0.66-fold \pm 0.14, $p < 0.05$ and 0.37-fold \pm 0.13, $p < 0.05$, respectively) (Figure 1.2). Thus it is concluded that administration of the inhibitor indeed led to a reduction of caspase-1 enzymatic activity. It was not possible to visualise bands for IL-1 β in the ipsilateral lumbar dorsal horn of either vehicle- or Ac-YVAD-CMK-treated animals. It is speculated that too little of the protein is present in these tissues, and that a dilution effect occurred owing to the size of the tissue in comparison to the size of DRG tissue.

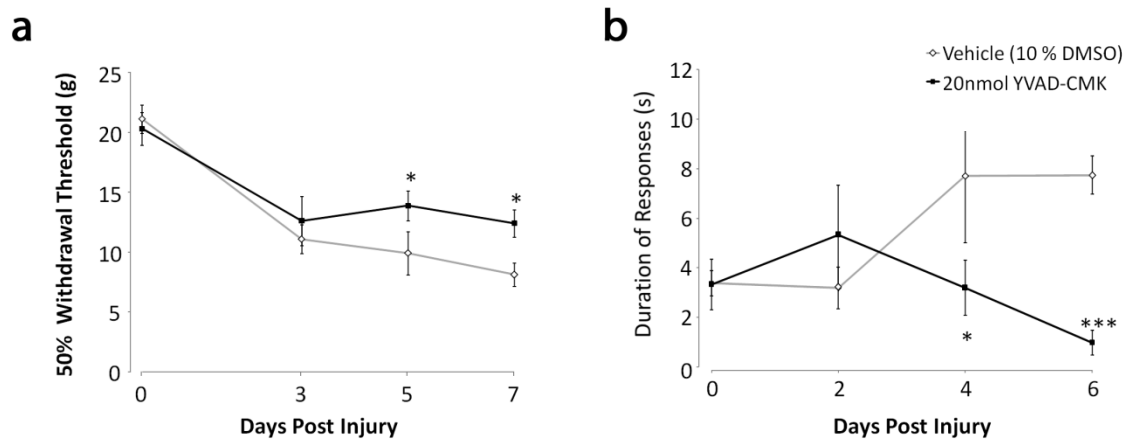


Figure 2.1: Caspase-1 inhibition attenuates L5 SNT-induced behavioural hypersensitivity

Daily intrathecal administration of 20 nmol Caspase-1 inhibitor Ac-YVAD-CMK attenuated L5 SNT-induced mechanical (a) and cold (b) hypersensitivity compared to vehicle-treated animals. Data represented as mean \pm SEM, $n=11-12$ animals per group. Two-way repeated measures ANOVA, followed by Dunnett's post-test, where *,*** $p<0.05$, 0.001 respectively, compared to vehicle-treated control responses on the same day. All behaviour was carried out blind to treatment.

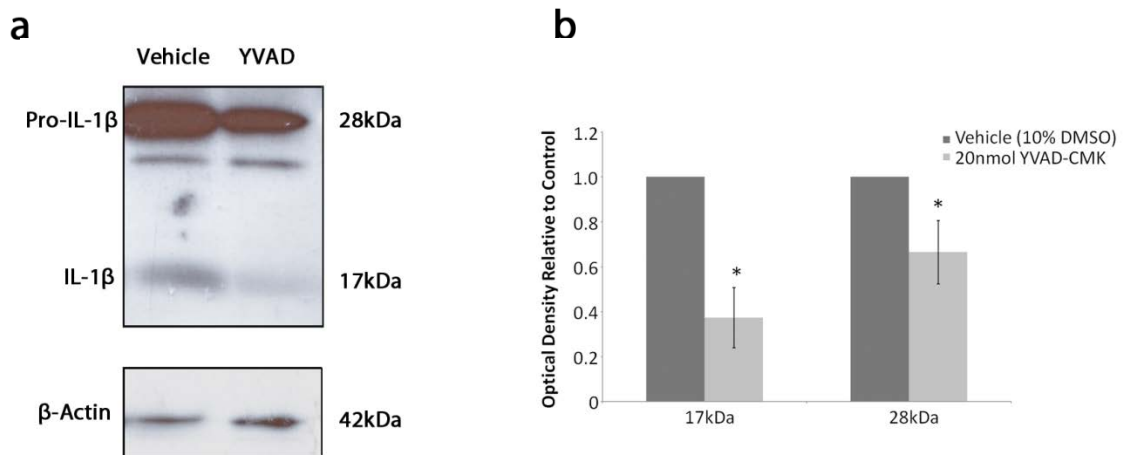


Figure 2.2: Caspase-1 inhibition reduces expression of IL-1 β

a: Western blot of the injured L5 DRG in rats 7 days after L5 SNT injury, with daily intrathecal injections of either vehicle (10% DMSO) or 20 nmol Ac-YVAD-CMK, a cell-permeant inhibitor of caspase-1, with quantification in **b**. Daily treatment with 20 nmol Ac-YVAD-CMK led to a reduction of both the immature pro-IL-1 β , and the mature active IL-1 β compared to vehicle-treated animals. Data represented as mean \pm SEM, $n=4$ animals per group (1 whole L5 DRG from each animal). One-way ANOVA, followed by Dunnett's post-test, where * $p<0.05$ compared to vehicle-treated control.

3.2. *Caspase-1 inhibition does not affect SNT-induced spinal microgliosis*

Immunostaining for IBA1 in the lumbar dorsal horn revealed no change in microglia numbers between the two groups, indicating that caspase-1 activity is not required for the development of microgliosis in the spinal cord. p38 is a MAP kinase, and phosphorylation of p38 is observed in microglia as they adopt an effector state following peripheral nerve injury. Its activation has been shown to be associated with peripheral nerve injury-induced behavioural hypersensitivity, and its inhibition has been shown to prevent the development of these behaviours. Immunostaining for phosphorylated p38 (p-p38) showed that microglial activation, according to this measure, was not altered by intrathecal pre-treatment with Ac-YVAD-CMK (Figure 2.3). Thus the attenuation of behavioural hypersensitivity observed in animals in which caspase-1 activity was inhibited is likely due to an effect downstream of the signalling processes leading to microglial activation.

Following peripheral nerve injury there are also changes in astrocytes within the spinal cord, including cellular hypertrophy and altered protein expression including an increase in GFAP. This occurs later than the development of microgliosis, generally reported to be significant 7 days after injury, and is much longer lasting. Immunostaining for the astrocyte marker GFAP revealed a significant decrease in the intensity of immunoreactivity in the ipsilateral superficial dorsal horn of animals treated with the caspase-1 inhibitor compared to vehicle treated animals (GFAP intensity of YVAD-treated rats $65.9\% \pm 2.80$, $p < 0.001$) (Figure 2.4).

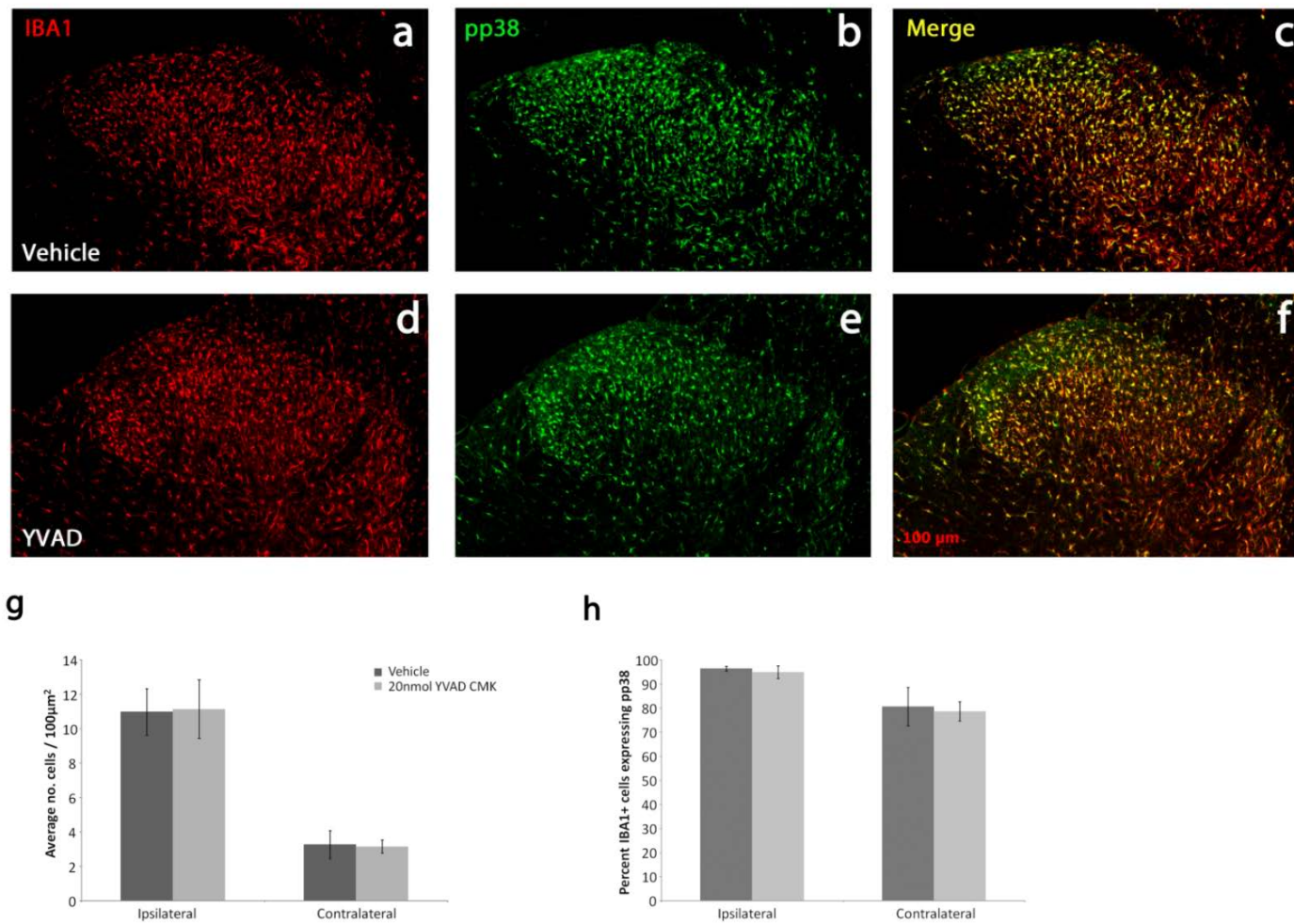


Figure 2.3: Caspase-1 inhibition does not alter L5 SNT-induced microgliosis: (figure legend on next page)

Figure 2.3: Caspase-1 inhibition does not alter L5 SNT-induced microgliosis:

a and d: Immunostaining for microglial marker IBA1 reveals no change in the number of microglia in the superficial dorsal horn of animals treated with caspase-1 inhibitor Ac-YVAD-CMK compared with vehicle-treated control animals. Quantified in **g. b and e:** Immunostaining for p-p38 shows no change in the number of p-p38 positive cells in the superficial dorsal horn of animals treated with caspase-1 inhibitor. **c and f:** merged images showing extensive co-localisation of IBA1 with p-p38. Scale bar 100 μm . **h:** Quantification showing the number of IBA1-positive cells expressing p-p38. No difference was observed between Ac-YVAD-CMK-treated animals, and vehicle-treated control animals. Data represented as mean \pm SEM, n=3 animals per group, with ≥ 3 spinal cord sections analysed per animal. One-way ANOVA with Dunnett's post-test.

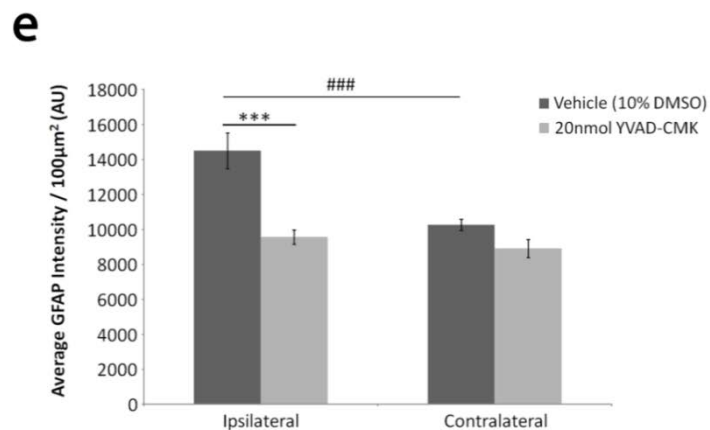
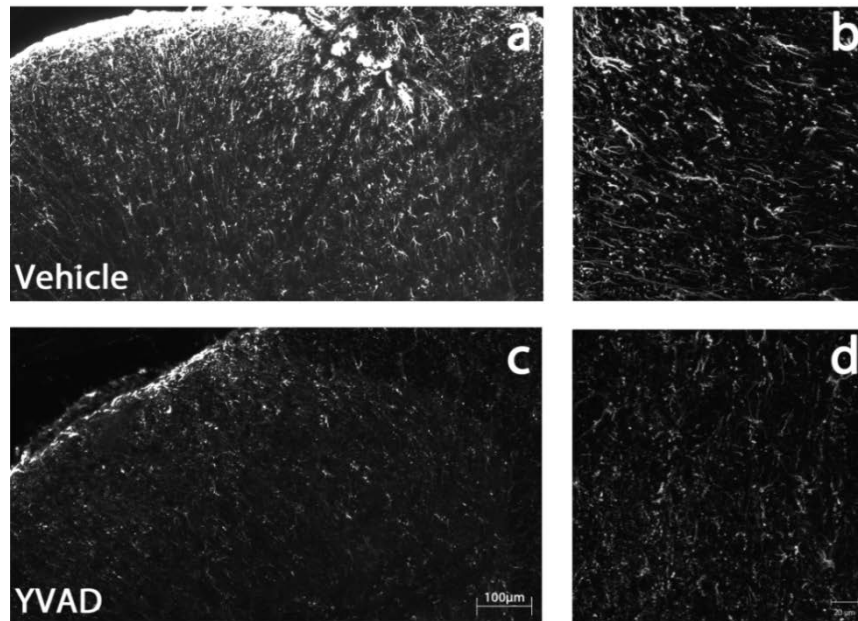


Figure 2.4: Caspase-1 inhibition reduces spinal cord astrocytosis after L5 SNT

Immunostaining for GFAP showed a significantly greater intensity of staining in the ipsilateral superficial dorsal horn in vehicle-treated animals than that observed in YVAD-treated animals. GFAP intensity was significantly greater in the superficial ipsilateral dorsal horn than the superficial contralateral dorsal horn of vehicle-treated animals. The intensity of GFAP was not significantly different in the ipsilateral and contralateral dorsal horns in YVAD-treated animals (**e**). Representative images, **a** and **c**, with higher-power confocal images, **b** and **d**. Scale bars: 100 µm, **a** and **c**, 20 µm, **b** and **d**. Data represented as mean \pm SEM, $n=3$ animals per group, with ≥ 3 spinal cord sections analysed per animal. Two-way ANOVA with Dunnett's post-test, where *******, **###** $p<0.001$ compared to vehicle-treated control animals, and compared to contralateral intensity of animals in the same treatment group, respectively.

3.3. Caspase-1 inhibition attenuates established hypersensitivity

We next asked whether caspase-1 inhibition could reverse or attenuate established pain-like behaviours. Animals received either vehicle (10% DMSO) or 20nmol Ac-YVAD-CMK intrathecally on days 5 to 8 post-surgery inclusive. Mechanical hypersensitivity was significantly attenuated in drug-treated animals on days 6 and 8 post-surgery ($p < 0.05$), and cold hypersensitivity was significantly reduced on day 6 post-surgery ($p < 0.05$). This effect was abolished on days 10 and 13 following cessation of treatment (Figure 2.5). No difference was observed in microglial number, co-localisation with p-p38, or GFAP immunoreactivity intensity, likely due to the fact that this was assessed 14 days post-surgery, 6 days after cessation of treatment (Figure 2.6).

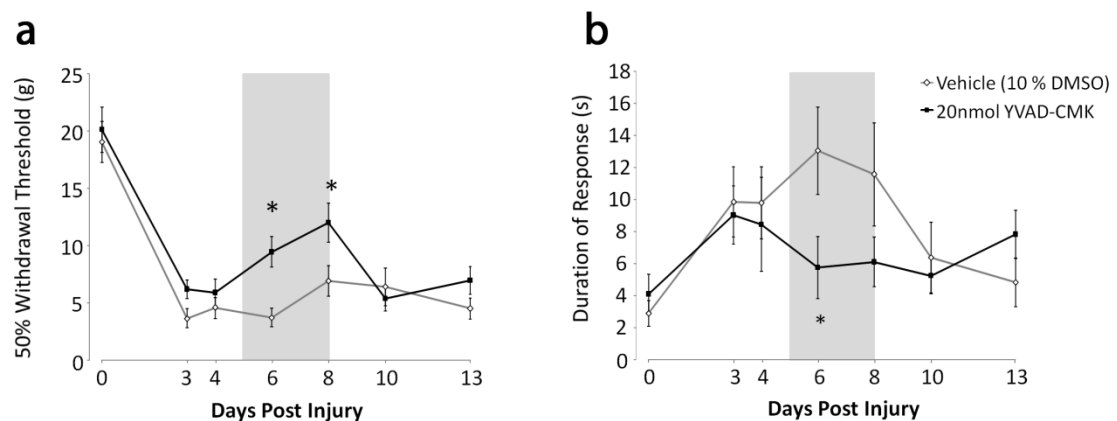


Figure 2.5: Caspase-1 inhibition attenuates established L5 SNT-induced behavioural hypersensitivity

Intrathecal administration of 20 nmol Caspase-1 inhibitor Ac-YVAD-CMK on days 5 to 8 post-surgery inclusive attenuated L5 SNT-induced mechanical **(a)** and cold **(b)** hypersensitivity compared to vehicle-treated animals. Shaded area on graphs indicate drug treatment. Data represented as mean \pm SEM, $n=6-7$ animals per group. Two-way repeated measures ANOVA, followed by Dunnett's post-test, where * $p < 0.05$ compared to vehicle-treated control responses on the same day. All behaviour was carried out blind to treatment.

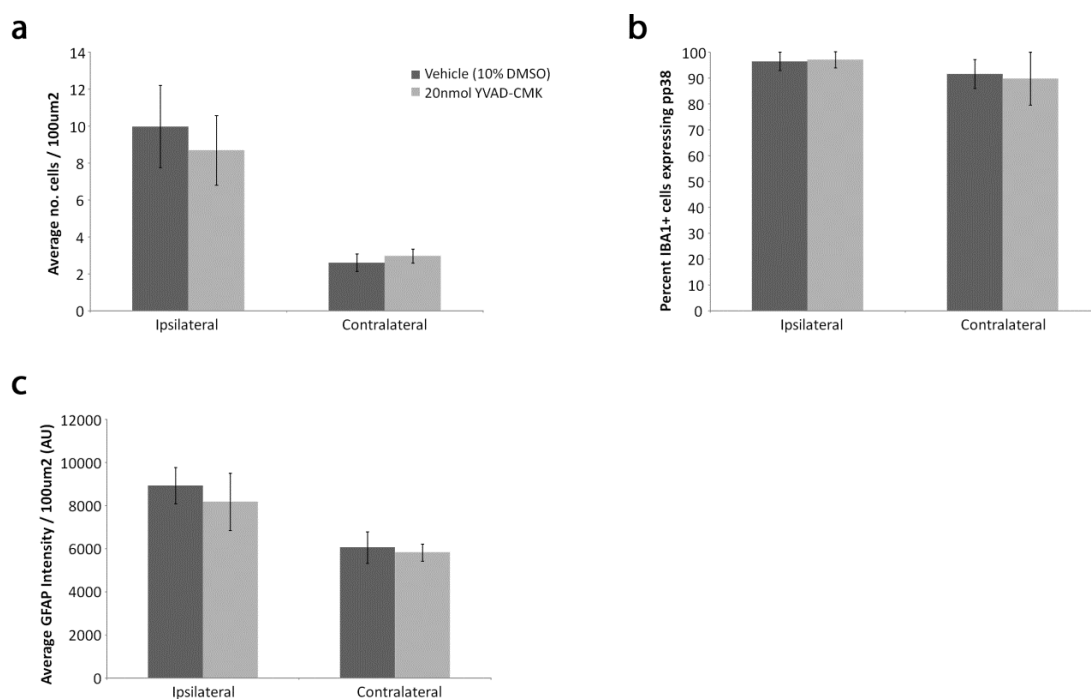


Figure 2.6: No change in microgliosis or astrocytosis in the superficial dorsal horns of animals 6 days after cessation of Ac-YVAD-CMK treatment

a: Immunostaining for microglial marker IBA1 reveals no change in the number of microglia in the superficial dorsal horn of animals treated with caspase-1 inhibitor Ac-YVAD-CMK compared to vehicle-treated control animals. **b:** No difference was observed in the amount of p-p38 co-localisation with IBA1 in superficial dorsal horns of drug-treated and vehicle-treated animals. **c:** No difference in GFAP immunoreactivity intensity was observed in the superficial dorsal horn of drug-treated animals and vehicle-treated animals. Data represented as mean \pm SEM, $n=3$ animals per group, with ≥ 3 spinal cord sections analysed per animal.

3.4. Caspase-1 inhibition prevents LPS-induced mechanical hypersensitivity

Intrathecal administration of LPS leads to hyperalgesia in the hind-paw (Cahill et al., 2003, Clark et al., 2006, Clark et al., 2010a). Rats were given a priming dose of LPS (2 µg in 10 µl 0.9% sterile saline), followed by a second intrathecal injection of LPS and either vehicle (10% DMSO in 0.9% sterile saline) or 20 nmol Ac-YVAD-CMK 24 hours later. Mechanical hypersensitivity was assessed 30 minutes, 1 hour, and 3 hours later. Following the final behavioural test animals were sacrificed and tissue collected. Vehicle-treated animals showed a significantly reduced mechanical withdrawal threshold from baseline at all time-points tested, whereas YVAD-treated animals did not develop a mechanical hypersensitivity compared to baseline levels at any time-point tested. Mechanical withdrawal thresholds were significantly greater in YVAD versus vehicle-treated animals at all time-points tested after LPS administration (Figure 2.7).

Immunohistochemical analysis revealed that although the total number of microglia in the superficial lumbar dorsal horn of YVAD-treated animals was not statistically different, there was a trend toward a decrease in number (average 4.4 ± 0.4 cells/100 µm² compared to 3.3 ± 0.3 cells/100 µm² in vehicle- and YVAD-treated animals respectively, $p=0.09$). The number of microglia expressing p-p38, indicating a state of activation, was significantly reduced in the superficial dorsal horns of animals treated with the caspase-1 inhibitor ($85.9\% \pm 3.0$ compared to $67.8\% \pm 4.3$ IBA1-positive cells expressing p-p38 in vehicle- and YVAD-treated animals respectively, $p<0.05$) (Figure 2.8). No difference was detected in the intensity of GFAP immunoreactivity between treatment groups (Figure 2.9).

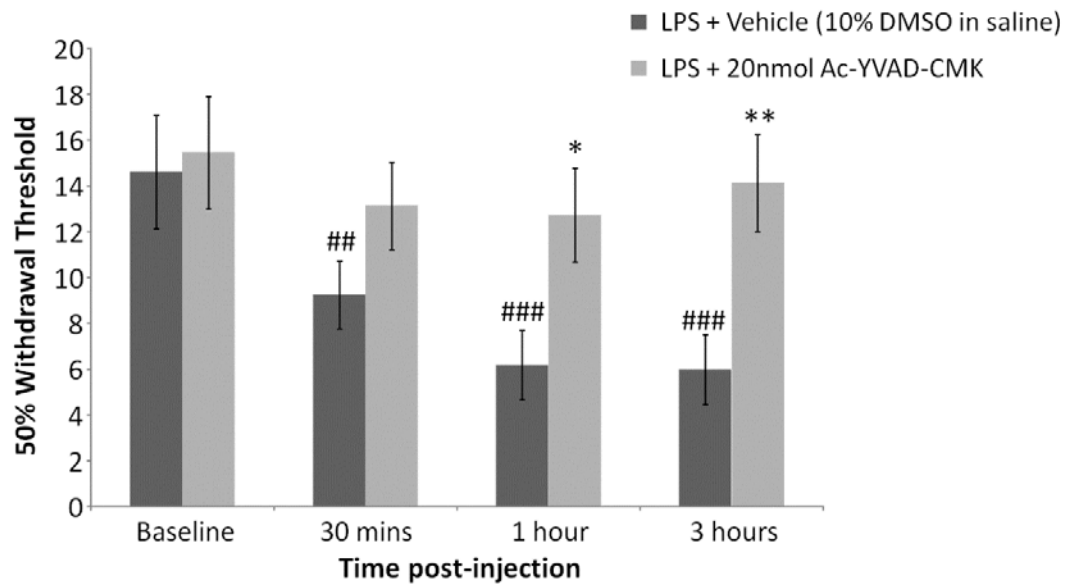


Figure 2.7: Caspase-1 inhibition prevents LPS-induced mechanical hypersensitivity

Intrathecal LPS produces a mechanical hyperalgesia of the hind-paw. Animals received a priming injection of LPS (2 µg in 10 µl 0.9% sterile saline), before receiving a second dose 24 hours later with a concomitant injection of either vehicle (10% DMSO in 0.9% sterile saline) or 20 nmol Ac-YVAD-CMK. Mechanical hypersensitivity was assessed 30 minutes, 1 hour, and 3 hours after the second LPS administration. Data represented as mean ± SEM, n=8 animals per group. Two-way repeated measures ANOVA, followed by Dunnett's post-test, where *, ** p<0.05, 0.01 compared to vehicle-treated animals' responses at the same time-point, where ##,###, p<0.01, 0.001 compared to baseline responses of the same treatment group. All behaviour was carried out blind to treatment.

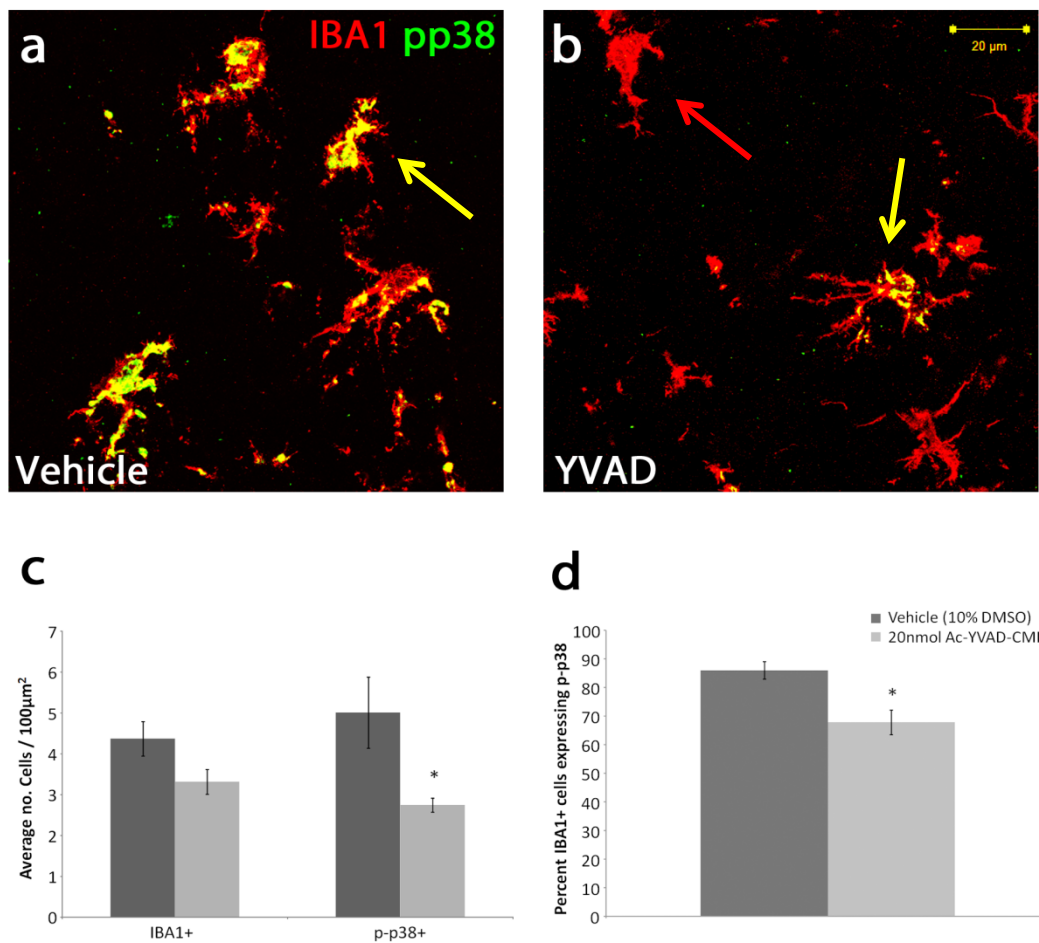


Figure 2.8: Caspase-1 inhibition reduces microglial activation following intrathecal LPS

Representative confocal microscope images of the superficial lumbar dorsal horns of vehicle- **(a)** and YVAD- **(b)** treated animals, IBA1 staining in the red channel, and p-p38 staining represented in the green channel. Yellow arrow indicates example cells which are double-labelled, whereas red arrows indicate example IBA1-positive cells which do not express p-p38. Scale: 20 μm . **c:** The number of microglia in the lumbar dorsal horn did not differ significantly between treatment groups, whereas the number of p-p38 positive cells was significantly reduced in animals treated with 20 nmol Ac-YVAD-CMK. **d:** The number of microglia expressing p-p38 was significantly reduced in YVAD-treated animals. Data represented as mean \pm SEM, $n=4$ animals per group, with ≥ 3 spinal cord sections analysed per animal. Two-tailed t test, where * $p<0.05$ compared to vehicle treated animals.

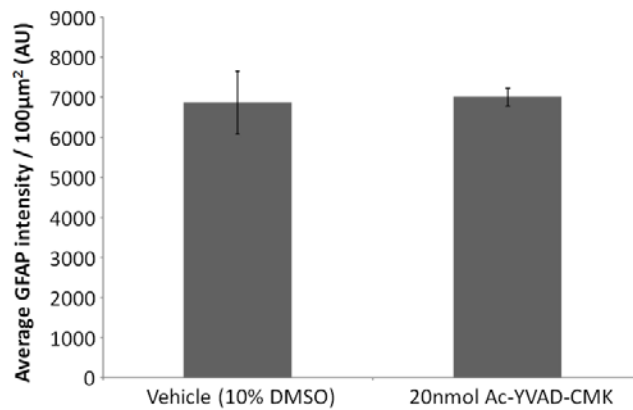


Figure 2.9: No change in GFAP immunoreactivity in the superficial dorsal horns of YVAD-treated animals following intrathecal LPS treatment

Immunostaining for GFAP, a marker of astrocytic activation, reveals no change in the superficial lumbar dorsal horn of animals treated with caspase-1 inhibitor Ac-YVAD-CMK compared to vehicle-treated control animals, following intrathecal LPS treatment. Data represented as mean \pm SEM, n=4 animals per group, with ≥ 3 spinal cord sections analysed per animal.

4. Discussion

There are several published reports showing that IL-1 β can elicit behavioural hypersensitivity (Falchi et al., 2001, Sung et al., 2004, Sung et al., 2005, Wolf et al., 2006, Gabay et al., 2011), as well as enhance AMPA- and NMDA-mediated currents and suppressing GABA-mediated currents (Kawasaki et al., 2008b). Moreover blockade of IL-1 β has been shown to attenuate pain-like behaviours in both inflammatory and neuropathic animal models of pain (Watkins et al., 1994, Sung et al., 2005, Gabay et al., 2011). Thus, in this study, the actions of IL-1 β were targeted upstream by inhibiting caspase-1 action with the cell-permeable peptide inhibitor Ac-YVAD-CMK.

In the present study, intrathecal pre-treatment and daily administration of YVAD-CMK decreased L5 SNT-induced mechanical and cold hypersensitivity. This appears to have been mediated, at least in part, by a reduction in IL-1 β as the amount of both inactive pre-cursor and active IL-1 β was significantly reduced in the injured DRG of YVAD-treated animals. This data is in disagreement with Kawasaki and colleagues who reported that inhibition of caspase-1 with YVAD-CMK did not affect L5 SNT-induced IL-1 β cleavage (Kawasaki et al., 2008a). However the group investigated the contribution of caspase-1 to SNT-induced changes at a much earlier timepoint than those investigated in this study. As reported in Chapter 1, increases in caspase-1 mRNA and protein expressions observed both in the ipsilateral lumbar dorsal horn and injured DRG did not become statistically significant until 7 days post-injury, and were decreasing again by 21 days post-injury; Kawasaki *et al.* reported no upregulation in the dorsal horn at 1 or 10 days post-injury. Additionally, YVAD was administered acutely with changes in IL-1 β expression investigated only 1 day post-injury whereas in the current study IL-1 β suppression was investigated 7 days after injury, with daily administration of YVAD. It is possible that the effect of the drug requires several treatments, which would explain the discrepancy between the two reports. This idea is enforced by the observation that differences in the behaviour between vehicle- and drug-treated animals did not become apparent until 4 days after injury.

IL-1 β released by microglia can activate glial IL-1R receptors in a positive feedback loop leading to the further activation of glia (Watkins and Maier, 1999). Thus, following daily treatment of L5 SNT-injured rats with either vehicle or YVAD, the number and activation state of spinal cord microglia was investigated. YVAD-treated animals did not show any difference in microglial number, or in the number of microglia expressing p-p38, a commonly used marker of microglial activation (Ji and Suter, 2007), suggesting that caspase-1 activity is likely to be a consequence, rather than cause, of SNT-induced microgliosis. Following peripheral nerve injury there are also changes in astrocytes within the spinal cord, including cellular hypertrophy and altered protein expression including an increase in GFAP. This occurs later than the development of microgliosis, generally reported to be significant 7 days after injury, and is much longer lasting (Colburn et al., 1999, Tanga et al., 2004, Tanga et al., 2006, Romero-Sandoval et al., 2008). Immunostaining for the astrocyte marker GFAP revealed a significant decrease in the intensity of immunoreactivity in the ipsilateral superficial dorsal horn of animals treated with the caspase-1 inhibitor compared to vehicle treated animals, suggesting a decrease in astrocytic activation. IL-1 β secreted from microglia is able to act on IL-1R expressed on neighbouring astrocytes, bringing about their activation (Watkins and Maier, 2003, Vallejo et al., 2010). In addition to IL-1 β cleavage, caspase-1 is able to cleave pro-IL-18 to the mature cytokine. A recent study showed that, following L5 SNT injury in rats, expression of IL-18 was significantly upregulated in microglia in the ipsilateral lumbar dorsal horn, while IL-18R expression was upregulated in astrocytes. IL-18 blockade attenuated injury-induced mechanical hypersensitivity, whereas intrathecal administration of IL-18 in rats caused mechanical hypersensitivity and astrocytic activation. Thus it is possible that, in addition to reducing IL-1 β activation of astrocytes, YVAD administration also reduced IL-18 activation of astrocytes. Unlike microglial activation following peripheral nerve injury, which is acute, astrocytic activation in animal models of pain occurs several days after the insult, and is much longer lasting (Romero-Sandoval et al., 2008). In the present study, although YVAD was administered daily after the injury, drug-treated animals did not show a difference in behavioural hypersensitivity until several days after injury. It is possible, therefore, that the anti-nociceptive effect of YVAD in

this study was mediated by the prevention of IL-1 β and IL-18-induced activation of astrocytes; a process that occurs several days after injury. This idea is supported by the finding that intrathecal YVAD treatment was also able to attenuate established hypersensitivity from 5 days post-injury. Astrocytosis was not investigated in these animals until several days after cessation of treatment, and so no difference in GFAP immunoreactivity was detected between vehicle- and YVAD-treated animals. It is also possible that IL-1 β and IL-18 act on dorsal horn neurons, which also have been shown to express these receptors to alter synaptic function, and YVAD treatment reduces this effect.

In the present study L5 SNT-induced hypersensitivity was not completely prevented, or reversed, by YVAD treatment. This could be due to an insufficient dose, though the amount of both inactive pre-cursor and active IL-1 β was significantly reduced in the injured DRG of YVAD-treated animals. Instead it is more likely that the behavioural data indicates that there are other mechanisms of importance contributing to L5 SNT-induced behavioural hypersensitivity. Blockade of IL-1 signalling has been shown to reduce, and even reverse, behavioural hypersensitivity in several animal models of pain (Maier et al., 1993, Safieh-Garabedian et al., 1995, Watkins et al., 1997, Wolf et al., 2006, Gabay et al., 2011, Nadeau et al., 2011). Caspase-1 has been known for several years to cleave inactive pro-IL-1 β to the mature cytokine, although there are reports that pro-IL-1 β can also undergo caspase-1-independent cleavage (Pintaux et al., 2007, Kawasaki et al., 2008a). Following L5 SNT it has been shown that IL-1 β cleavage can occur through the action of matrix metalloproteases (MMP) MMP-9 and MMP-2 during early and later phases of neuropathic pain respectively (Kawasaki et al., 2008a). Thus, although IL-1 β cleavage was reduced by YVAD treatment, it is likely that other mechanisms of IL-1 β cleavage persisted contributing to the generation and maintenance of behavioural hypersensitivity.

Caspase-1 inhibition has been previously investigated in inflammatory models, including the carrageenan model (Cunha et al., 2000) and hindpaw injection of complete Freund's adjuvant (Samad et al., 2001), though in both these studies,

caspase-1 inhibition was peripheral. In the present study we investigated the effect of caspase-1 inhibition on LPS-induced inflammation. This particular model of inflammation was chosen because the LPS is applied directly to the spinal cord to produce a sterile central inflammation, isolated from any peripheral immune response. Additionally it relates closely to the *in vitro* experiments carried out in Chapter 1. A priming dose of LPS was administered 24 hours before the second dose, after which behavioural testing was carried out. LPS produced bilateral hindpaw mechanical hypersensitivity within 30 minutes of administration in vehicle-treated rats. 1 hour after administration the hindpaw mechanical withdrawal threshold was decreased further. Concomitant treatment of Ac-YVAD-CMK with the LPS prevented this reduction in hindpaw withdrawal threshold. Immunohistochemical analysis revealed that although the number of microglial cells in the superficial lumbar dorsal horn of animals treated with the caspase-1 inhibitor was not significantly altered, the number of microglial cells expressing p-p38 was significantly reduced in YVAD-treated animals indicating that YVAD treatment led to a reduced microglial activation in response to LPS. No difference was observed in the intensity of GFAP immunoreactivity, though astrocytic activation would not be expected at such an early timepoint following the inflammatory stimulus, and has not been previously reported.

Data presented here suggest that caspase-1 plays a role in the generation and maintenance of behavioural hypersensitivity in the L5 SNT model of neuropathic pain and also the central inflammatory model of LPS-induced hypersensitivity. However it has been shown that the relative contribution of caspase-1 to the development and maintenance of neuropathic pain differs between animal models such that the non-specific caspase-1 inhibitor Z-VADFMK was maximally effective in ddC- (HIV therapy) model, marginally effective in vincristine-induced hyperalgesia model, but was ineffective in STZ-induced model of neuropathic pain (Joseph and Levine, 2004). Subsequent work will examine other proteins of the inflammasome, and their role in persistent pain states.

Chapter 3: The Role of the Inflammasome Adaptor Protein ASC in Persistent Pain States

1. Introduction

Data presented thus far show that caspase-1 signalling contributes to mechanical and cold hypersensitivity in the L5 SNT model of neuropathic pain, and to a greater extent following intrathecal LPS administration as a model of central inflammation. Several studies have shown a requirement for ASC in pro-caspase-1 activation. ASC is a 22kDa protein, consisting of two homotypic protein interaction domains; an amino-terminal PYRIN domain and a carboxy-terminal CARD domain. Several lines of evidence suggest that this protein is necessary for NLRP3 inflammasome mediated secretion of IL-1 β . Immunodepletion of ASC in THP-1 cell extracts has been shown to block caspase-1 activation, and subsequent IL-1 β maturation following LPS stimulation (Martinon et al., 2002). Additionally LPS-primed macrophages from ASC^{-/-} mice fail to activate caspase-1, and subsequently release IL-1 β and IL-18. However TNF- α and IL-6, also pro-inflammatory cytokines, were released from cells normally. *In vivo* experiments show that mice deficient in ASC do not demonstrate elevated serum IL-1 β and IL-18 following systemic LPS challenge (Mariathasan et al., 2004, Yamamoto et al., 2004).

Given that caspase-1 inhibition reduced behavioural hypersensitivity in a model of neuropathic pain and inflammation, we next explored the effect of ASC inhibition on behavioural hypersensitivity in animal models of pain.

2. Methods

2.1. *Animals and surgery*

Adult (250 – 300g) male Wistar rats (Harlan, UK), adult (10 – 12 weeks) male C57Bl/6 mice (Harlan, UK), and adult (10 – 12 weeks) male and female ASC-/- and wildtype littermates (sourced from Genentech), bred on a C57Bl/6 background, were used in accordance with UK Home Office regulations (Animals Scientific Procedures Act 1986).

Genotyping: A tissue sample of mouse pups (postnatal days 10 – 14) was obtained by ear punch (Harvard Apparatus, UK), which was also used for identification purposes. The tissue incubated with 30 µl lysis buffer (consisting of 67 mM Tris (Sigma), 16 mM (NH₄)₂SO₄ (Sigma), 6.7 mM MgCl₂ (Sigma), 0.2%w/v β-mercaptoethanol (Sigma), 0.4 mg/ml Proteinase K (VWR)) at 55°C for 1 hour, followed by 10 minutes at 95°C. Samples were briefly vortexed, then centrifuged, with the resultant supernatant containing the DNA used for PCR. Genotype was determined by a polymerase chain reaction (PCR). The master mix solution consisted of 1.5 mM MgCl₂ (Promega), 20% v/v 5x Reaction Buffer (Promega) 0.2mM dNTP (Promega), 50 units/ml Taq DNA polymerase (Promega), 0.1 µM primers x 3 (Sigma), 0.5 µl DNA sample.

Primer sequences:

sm99-gASC (forward): CTA GTT TGC TGG GGA AAG AAC

sm105-cASC (reverse): CTA AGC ACA GTC ATT GTG AGC TCC

sm141-NEOseq6 ASC (forward): AAG ACA ATA GCA GGC ATG CTG G

Each reaction consisted of 0.5 µl DNA, and 24.5 µl master mix. The PCR reaction was performed in TC412 Flexigene thermal cycle (Techne, UK). The conditions for the PCR reaction were as follows: denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds, and polymerisation at 72°C for 1 minute. This was followed by a final extension step at 72°C for 10 minutes. The expected size of the products were 450bp for wildtype, 260bp for knockout, and 450 and 260bp for heterozygotes.

Visualisation: To visualise the PCR products, gel electrophoresis of the samples was performed, using a 2% agarose gel containing 100 µg/ml

ethidium bromide (Sigma) for visualisation. Gels were viewed using GeneGenius system (Syngene), and imaged using GeneSnap (Syngene).

L5 SNT: Rats received L5 SNT injury using the same method as described in Chapter 1.

Partial sciatic nerve ligation: Under isoflurane anaesthesia, the left sciatic nerve was exposed at mid-thigh level. A 5-0 silk suture was used to tightly ligate approximately a third to half of the nerve. The wound was closed using two wound clips. No post-surgical analgesia was given in order to avoid potential modulation of the nociceptive and immune system of the animals. The general health of the animals was monitored daily after the surgery.

Drugs and delivery: CRID3 (CP-456,773) (Pfizer, Ltd., UK) was administered as 25 nmol dissolved in 20 μ l 10% DMSO in sterile saline. Under isoflurane anaesthesia a 26G insulin syringe (Myjector U-100 Terumo) was inserted between the L5 and L6 vertebrae, where the spinal cord consists mainly of spinal roots. A volume of 20 μ l was injected, after which the needle was slowly removed. The accuracy of each injection was ensured through the observation of an injection-induced tail-flick (Mestre et al., 1994).

As described in Chapter 1, animals were either sacrificed by terminal anaesthesia and transcardially perfused with either 4% paraformaldehyde plus 1.5% picric acid for histology, or saline alone for Western blot analysis. Tissue used for histology: Lumbar spinal cords were excised and post-fixed overnight, dorsal root ganglia (DRG) were post-fixed for 3-4 hours. Tissue was cryoprotected in 20% sucrose in 0.1 M Phosphate Buffer (PB) for 4-7 days before OCT embedding. Tissue used for Western blot analysis: Lumbar spinal cords were excised and quadrisectioned. Lumbar spinal cords and DRG were snap-frozen in liquid nitrogen and stored at -80°C.

2.2. Behavioural testing

Animals were tested in a temperature stable room during the light period of their day/night cycle, at the same time every day. Animals were placed in a polymethyl methacrylate box with a wire grid bottom and allowed to habituate

for a period of 15-30 minutes before testing. Animals were trained once for each behavioural test, and then two baseline tests were performed on different days.

Assessment of mechanical withdrawal thresholds: Mechanical withdrawal thresholds were assessed using calibrated von Frey hairs (Ugo Basile) according to the “up-down” method (Chaplan et al., 1994), as described in Chapter 1. The 50% withdrawal threshold was then calculated using the method described by Dixon (Dixon, 1980).

Assessment of cold hypersensitivity – acetone test: For measurement of cold hypersensitivity, a drop of acetone was applied to the plantar surface of the rat hindpaw, as described in Chapter 1. 3 tests were carried out, at least 10 minutes apart from each other, and an average response was recorded.

Assessment of heat thresholds - Hargreaves test: The noxious heat threshold of the hind paw was determined using Hargreaves Plantar Test (Hargreaves et al., 1988). Animals were placed in acrylic cubicles (8 x 5 x 10 cm) atop a uniform glass surface, and allowed to habituate before testing. An infrared light source was directed to the plantar surface of the hind paw, and the latency to withdraw was recorded. To prevent tissue injury, the maximum stimulus duration was 20 seconds. Three responses were recorded for each hind paw, and an average response for each taken.

Hot / cold plate test: Response to continued noxious thermal stimulation of the paws was assessed using an Incremental Hot / Cold Plate (IITC Life Sciences, Woodland Hills, CA, USA), set at 4, 50, and $52 \pm 0.1^{\circ}\text{C}$, with latency to lick or flinch the hindpaws recorded. To avoid tissue injury animals were removed after 20 seconds if no response was observed. Three responses were recorded, and an average response taken.

Locomotor Function: Locomotor function was assessed using an accelerating RotaRod (Ugo Baile), set to accelerate from 2 to 40 rpm over a period of 560 seconds. Latency to fall was recorded. Three responses were recorded, and an average response taken.

All behavioural testing was carried out blind to treatment, or genotype.

2.3. Western blot

Tissues were homogenised in the same way as described in Chapter 1. Protein concentration of all samples was determined using a BCA Protein Assay kit (Thermo Scientific). Lysates were normalised to 35 µg protein per sample. Proteins (35 µg lysate per well) were separated using SDS-PAGE and transferred to 0.2 µm pore size PVDF membranes (Millipore). Membranes were blocked in 10% skimmed milk in PBS-T for 45 minutes before overnight incubation with primary antibody at 4°C as already described in Chapter 1. Species appropriate HRP-conjugated secondary antibody (1:10,000, Amersham) was applied the subsequent day for 1 hour at room temperature. Membranes were visualised using ECL-prime reagent for 5 min (GE Healthcare) before detection by autoradiography. For visualisation of protein loading control, the membranes were stripped using a β -mercaptoethanol buffer as described in Chapter 1, before incubation with primary antibody.

Antibodies used: Rabbit anti-ASC (1:1000, Alexis Biochemicals), mouse anti- β -actin (1:15,000, Sigma), donkey anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:20,000, GE Healthcare), donkey anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:20,000, GE Healthcare).

Blots were analysed by determining mean intensity of each band using a fixed box size for all. The background intensity for each blot was then subtracted from all values obtained. All quantifications were then normalised against β -actin loading control. All blots were then compared to the mean band intensity for control-treated samples or naive animals. Analysis was carried out using Quantity One 1-D Analysis Software version 4.6.9 (Biorad).

2.4. Histology

Lumbar spinal cords were cryostat cut in 20 µm sections and thaw-mounted onto glass slides. When slides were fully dry (at least 1 hour after tissue mounting), they were stored in cryoprotectant (30% ethylene glycol, 30% glycerol, 40% PBS) at -20°C until required.

Immunohistochemistry was carried out according to the methods described in Chapter 1. Primary antibodies used: Rabbit anti-GFAP (1:1000, DAKO), rabbit anti-IBA1 (1:1000, WAKO), rabbit anti-p-p38 (1:100, Cell Signalling). Tyramide signal amplification was used to visualise p-p38. Secondary antibodies: anti-mouse or anti-rabbit Alexa Fluor 488 (1:1000, Stratech), anti-mouse or anti-rabbit Alexa Fluor 546 (1:1000, Stratech), ExtrAvidin FITC (1:200, Sigma). Specificity of staining was determined by omission of the appropriate primary antibody as a negative control. Slides were mounted on coverslips using Vectashield mounting medium with DAPI (Vectorlabs) and visualised using a Zeiss Axioplan 2 fluorescent microscope.

Quantification:

Cell counts / colocalisation: 3 $100\ \mu\text{m}^2$ boxes were drawn over the superficial L5 dorsal horn, ipsilateral and contralateral to the injury. Cells which were associated with a nuclear stain, within the confines of the box, were counted. An average count from the 3 boxes was taken. At least 3 sections per animal were analysed. Counts were performed manually. Analysis was carried out blind to treatment group / genotype.

GFAP intensity: All GFAP images had been taken on the same day, using the same exposure. As for the cell counts, 3 $100\ \mu\text{m}^2$ boxes were drawn over the superficial L5 dorsal horn, ipsilateral and contralateral to the injury. A $20\ \mu\text{m}^2$ box was drawn in the white matter, and was used to generate a background intensity value. The intensity of the 3 boxes, minus the background intensity value, was averaged. At least 3 sections per animal were analysed. All analysis was carried out blind to genotype. Analysis was carried out using AxioVision LE version 4 software.

2.5. Statistical analysis

All data are represented as mean \pm SEM where appropriate. Western blot, and histology data were analysed using one-way ANOVA followed by Dunnett's post-test. Behavioural data were analysed using two-way repeated measures ANOVA followed by Dunnett's post-test. Where data were not normally distributed, ANOVA tests were performed on ranked data. All analysis was carried out using the statistical package SigmaPlot for Windows, version 12.

3. Results

3.1. ASC inhibition attenuates established L5 SNT-induced behavioural hypersensitivity

To investigate the role of the inflammasome upstream of caspase-1 activation, we investigated the effect of inhibition of the adaptor protein ASC. The Cytokine Release Inhibitory Drug (CRID), CRID3 has recently been shown to inhibit IL-1 β maturation and release by targeting ASC oligomerisation (Coll and O'Neill, 2011). Animals received either vehicle (10% DMSO) or 25 nmol CRID3 intrathecally on days 5 to 8 post-surgery inclusive. There was a small but significant reduction in mechanical hypersensitivity drug-treated animals on days 7 and 8 post-surgery ($p < 0.05$). This effect was abolished on days 9, 10, and 13 following cessation of treatment. However no difference in cold hypersensitivity was observed (Figure 3.1).

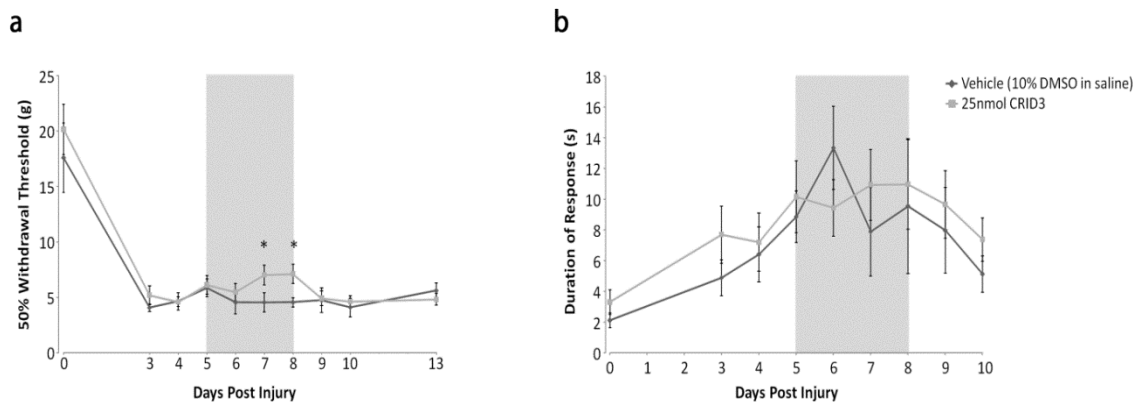


Figure 3.1: ASC inhibition attenuates established L5 SNT-induced mechanical but not cold hypersensitivity

Intrathecal administration of 25 nmol CRID3 on days 5 to 8 post-surgery inclusive attenuated L5 SNT-induced mechanical (a) but not cold (b) hypersensitivity compared to vehicle-treated animals. Shaded area on graphs indicate drug treatment. Data represented as mean \pm SEM, $n=7-8$ animals per group. Two-way repeated measures ANOVA, followed by Dunnett's post-test, where * $p < 0.05$ compared to vehicle-treated control responses on the same day. All behaviour was carried out blind to treatment.

3.2. Acute responses to noxious stimuli of ASC^{-/-} mice are comparable to those of wildtype littermates

The role of the adaptor protein ASC in animal models of pain was investigated further using a strain of ASC^{-/-} mice. The generation of these mice has been described previously, and is illustrated in Figure 3.2 (Mariathasan et al., 2004). Immunohistochemical analysis shows that ASC^{-/-} mice express microglial marker IBA1 normally, however ASC was not detected (Figure 3.3 a). Western blot analysis confirmed that ASC^{-/-} mice do not express ASC protein (Figure 3.3 b).

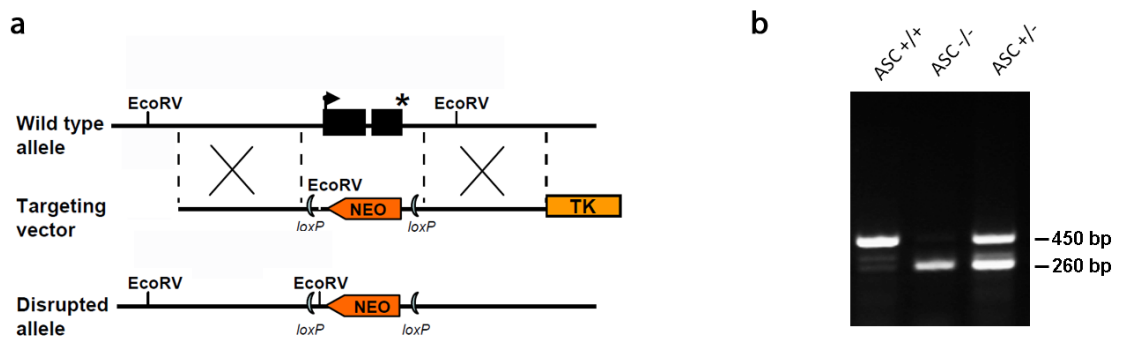


Figure 3.2: Targeted disruption of the murine ASC gene

a: Schematic demonstrating the targeted disruption of the murine ASC gene. Exons (closed black boxes) encoding the ASC gene were replaced with a neomycin resistance cassette. X up- and downstream of this cassette indicate homology arms. TK indicates a thymidine kinase negative selection marker, outside the region of sequence similarity between the wildtype allele and targeting vector. Adapted from (Mariathasan et al., 2004). **b:** Representative agarose gel of PCR products from genotyping, with wildtype mice yielding a 450bp product, knockout mice yielding a 260bp product, and heterozygote mice yielding both products.

The effect of ASC gene deletion on sensory processing pathways has not previously been investigated. Thus the behaviour of ASC^{-/-} mice and wildtype littermates was assessed in a range of tests to determine if there was any difference in acute pain processing in response to peripherally-applied mechanical and thermal stimuli. No significant difference was observed between ASC^{-/-} mice and wildtype littermates in withdrawal latency to noxious thermal stimuli in the hot / cold plate test at any of the temperatures tested; 4, 50, and 52°C. In line with these findings, no difference was observed in withdrawal latency to a gradual radiant heating of the plantar surface in the Hargreaves test. ASC^{-/-} mice also exhibited equivalent withdrawal thresholds to von Frey mechanical stimuli. No deficit in motor function was observed, as observed by the RotaRod test (Figure 3.4).

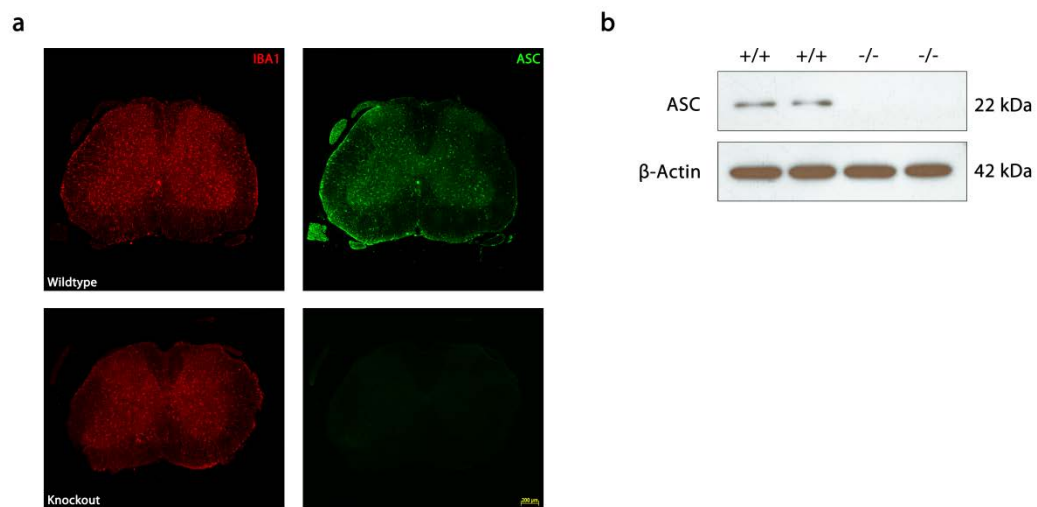


Figure 3.3: ASC ^{-/-} mice are deficient in ASC protein expression

a: ASC^{-/-} mice express microglial marker IBA1 in the spinal cord normally, but do not express ASC. Scale 200 μm. **b:** Western blot analysis of spinal cord lysates confirms that ASC^{-/-} mice do not express ASC.

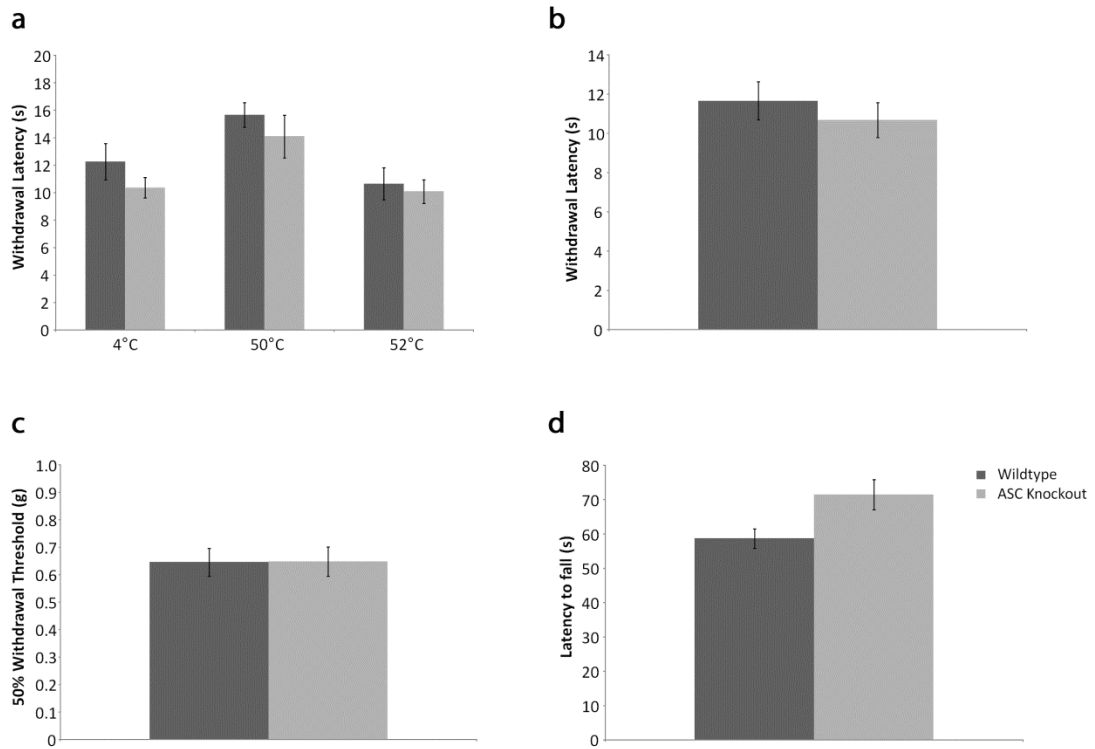


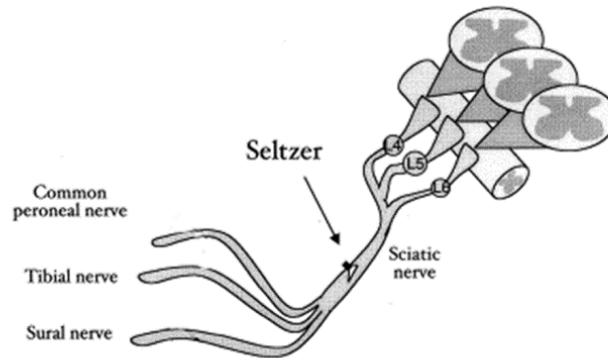
Figure 3.4: ASC^{-/-} mice display normal responses to acute thermal and mechanical noxious stimuli

The withdrawal thresholds of naïve ASC^{-/-} and ASC^{+/+} mice were assessed in the hot / cold plate test **(a)**, the Hargreaves test **(b)**, and the von Frey test **(c)**. Motor function was assessed using the RotaRod test **(d)**. Data represented as mean \pm SEM, n=11-12 mice per group. Students t test. All data non-significant.

3.3. Partial sciatic nerve ligation causes behavioural hypersensitivity and spinal microgliosis in mice

Baseline mechanical responses in adult male C57Bl/6 mice were assessed prior to any surgery. The diagram in Figure 3.5 demonstrates the injury. Following partial sciatic nerve ligation (PSNL) the change in behavioural sensitivities were assessed intermittently over a ten-day period, to ensure that the animals developed neuropathic pain-like behaviours. PSNL caused a significant decrease in hindpaw 50% withdrawal threshold to von Frey hairs, observed from the first time-point assessed; 3 days. This mechanical hypersensitivity lasted for the duration of the study and was only observed in the hindpaw ipsilateral to the injury, whereas contralateral thresholds were unaffected (Figure 3.2 b).

a



b

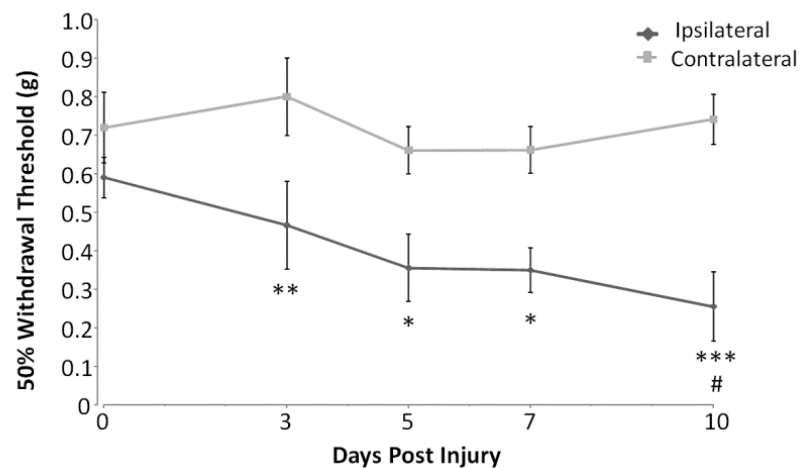


Figure 3.5: Partial sciatic nerve ligation causes lasting hindpaw mechanical hypersensitivity

a: The sciatic nerve is exposed at mid-thigh level. A 5-0 silk suture is used to tightly ligate $\frac{1}{3}$ to $\frac{1}{2}$ of the nerve. Diagram adapted from (Shields et al., 2003). **b:** PSNL caused a significant decrease in hindpaw 50% withdrawal threshold to von Frey hairs using the Up-Down method in the ipsilateral hindpaw, observed from the first time-point assessed, lasting up to ten days. Contralateral hindpaw withdrawal thresholds were unaffected. Data represented as mean \pm SEM, $n=8$ mice. Two-way repeated measured ANOVA, followed by Dunnett's post-test, where *, **, *** $p<0.05$, 0.01, 0.001 compared to baseline responses.

10 days after PSNL a robust microgliosis was observed using immunohistochemical techniques, visualised using the microglial marker IBA1. The number of IBA1-positive cells in the ipsilateral dorsal horn was significantly increased compared to the contralateral dorsal horn (4.18-fold increase \pm 0.74 compared to contralateral dorsal horn). As observed in the rat after L5 SNT injury, the number of ASC-positive cells was significantly increased in the ipsilateral superficial lumbar dorsal horn compared to the contralateral dorsal horn (4.39-fold increase \pm 0.84 compared to contralateral dorsal horn). Again, as observed in the rat, there was extensive co-localisation of ASC with microglial marker IBA1 (Figure 3.6).

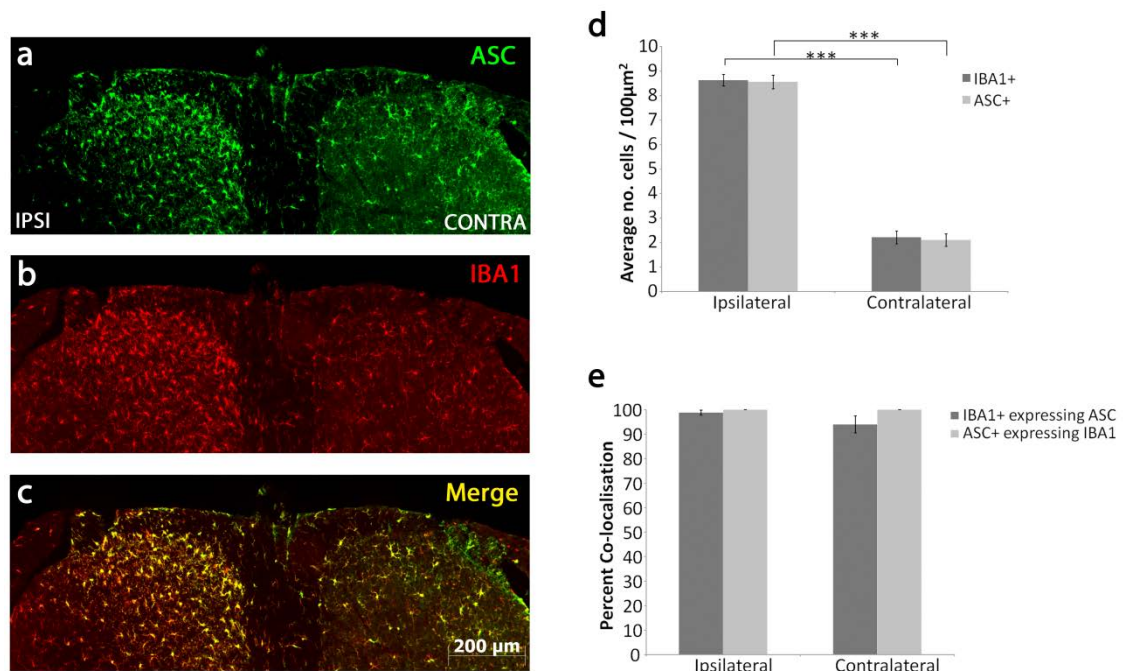


Figure 3.6: Partial sciatic nerve ligation causes spinal microgliosis in the mouse Lumbar spinal cord of an animal 10 days post-surgery, immunostained for ASC (**a**), and microglia (**b**) using IBA1 antibody. Merged image shown in **c**. Scale 200 μ m. **d**: The number of ASC-positive and IBA1-positive cells in the ipsilateral superficial dorsal horn was significantly greater than that observed in the contralateral dorsal horn, 10 days post-surgery. **e**: Co-localisation of ASC- and IBA1-positive cells is 100%. Data represented as mean \pm SEM, n=4 animals, with \geq 3 spinal cord sections analysed per animal. Paired t-test, where *** $p < 0.001$ compared to contralateral superficial dorsal horn.

3.4. Partial sciatic nerve ligation causes comparable behavioural hypersensitivity and immunohistochemical marker changes in ASC-/- mice and wildtype littermates

Baseline mechanical responses in adult ASC-/- and ASC+/+ mice were assessed prior to surgery. PSNL caused a significant and lasting decrease in ipsilateral hindpaw 50% withdrawal threshold to von Frey hairs. Additionally there was a significant decrease in withdrawal latency of the hindpaw in the Hargreaves test. No difference was detected in the responses of knockout and wildtype animals, however (Figure 3.7). 14 days after injury a significant increase in the number of microglia in the superficial ipsilateral lumbar dorsal horn was observed, compared to the uninjured contralateral side. This increase was observed in both ASC-/- and wildtype littermates, with no difference between the two. Additionally there was no difference observed in the level of activation of microglia between the two genotypes, as assessed by co-localisation with p-p38. As well as an ipsilateral increase in microglial number, there was an increase in astrocyte activation as assessed by a significant increase in the intensity of GFAP immunofluorescence. No difference was observed between ASC-/- and wildtype littermates (Figure 3.8).

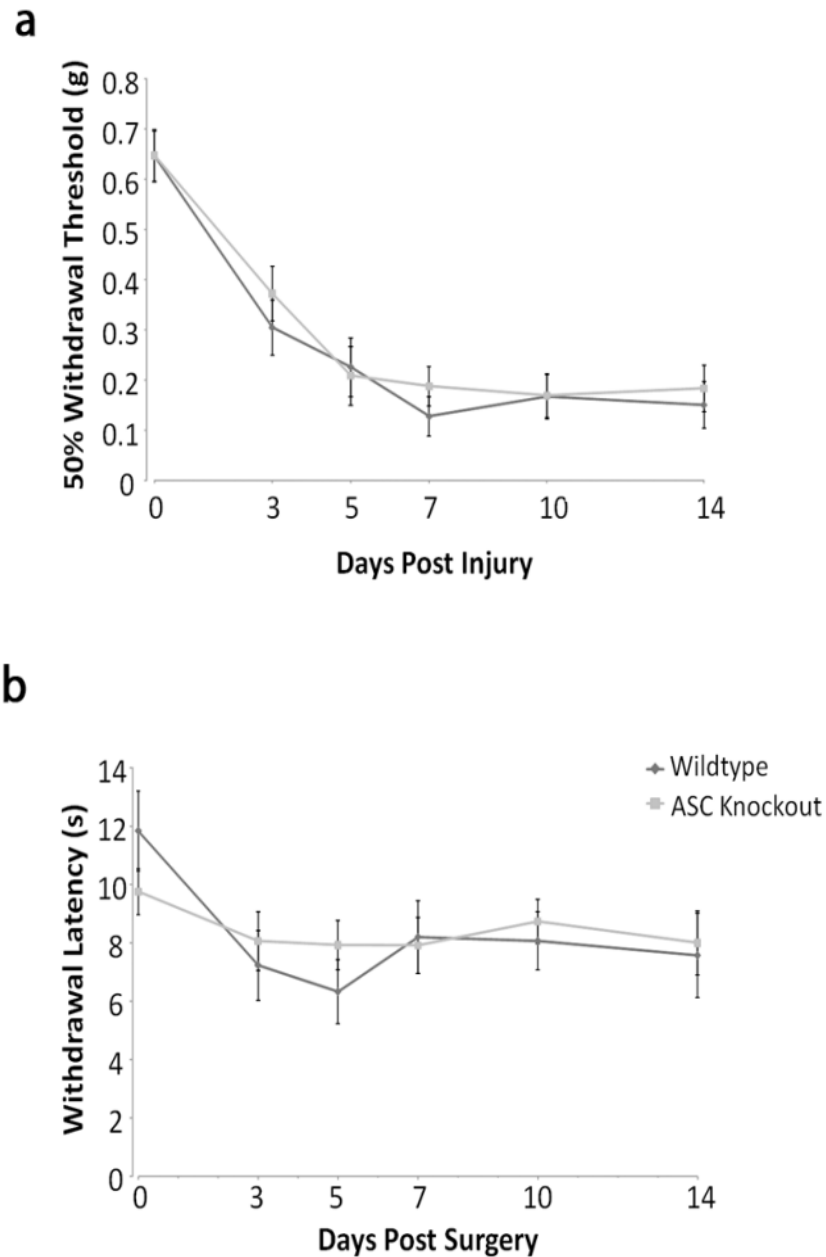


Figure 3.7: Partial sciatic nerve ligation causes comparable behavioural hypersensitivity in ASC^{-/-} mice and wildtype littermates

a: PSNL caused mechanical hypersensitivity in the ipsilateral hindpaw in both ASC^{-/-} mice and wildtype littermates, with no discernible difference between the two genotypes. **b:** PSNL caused a decrease in withdrawal latencies of the ipsilateral hindpaw to a radiant heat stimulus in the Hargreaves plantar test. There was no discernible difference in the withdrawal latencies of ASC^{-/-} and wildtype littermate mice at any timepoint tested. Data expressed as mean \pm SEM, n=11-14 mice per group. Two-way repeated measures ANOVA, with Dunnett's post-test.

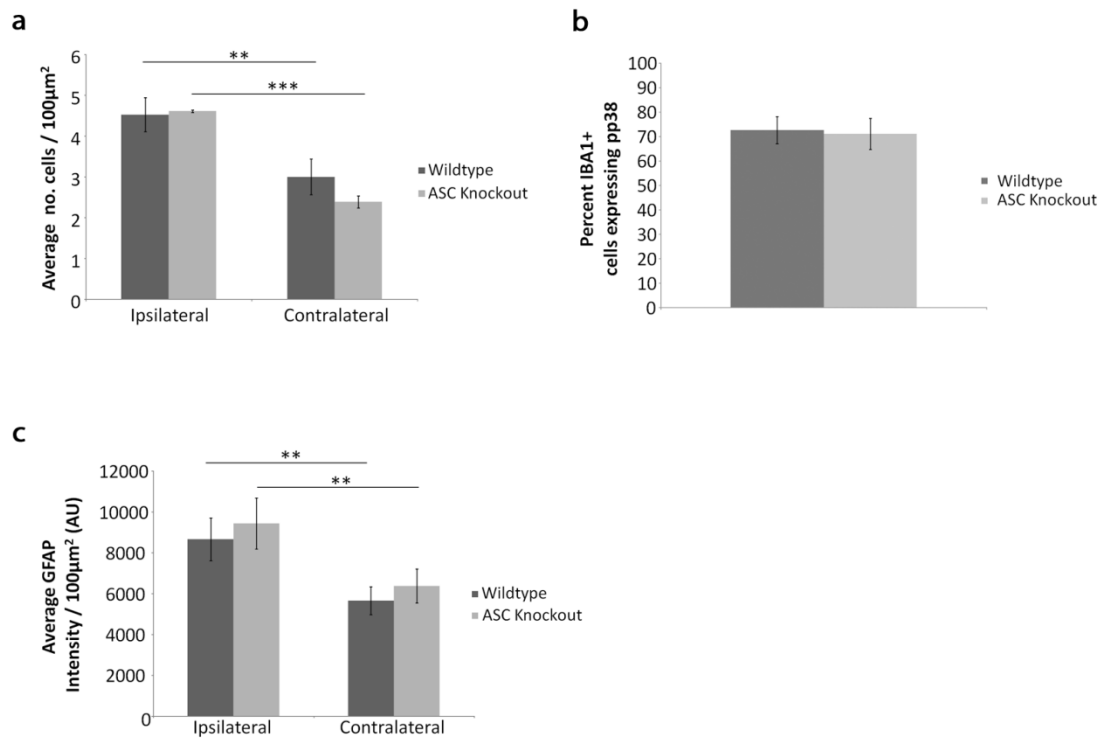


Figure 3.8: No difference in the level of microgliosis or astrocytosis in the superficial dorsal horns of ASC^{-/-} mice and wildtype littermates following partial sciatic nerve ligation

a: Immunostaining for microglial marker IBA1 reveals a significant increase in the number of microglia in the superficial ipsilateral lumbar dorsal horn of both ASC^{-/-} and wildtype littermates compared to the contralateral side. No significant difference between the two phenotypes was observed. **b:** No difference was observed in the amount of p-p38 co-localisation with IBA1 in superficial dorsal horns of ASC^{-/-} mice compared to wildtype littermates. **c:** Immunostaining for astrocyte marker GFAP reveals a significant increase in immunofluorescence in the superficial ipsilateral lumbar dorsal horn compared to the contralateral side in both ASC^{-/-} and wildtype littermate mice. No significant difference between the two phenotypes was observed. Data represented as mean \pm SEM, n=4 animals per group with ≥ 3 spinal cord sections analysed per animal. Two-way repeated measures ANOVA with Dunnett's post-test.

3.5. *ASC deficiency attenuates LPS-induced hindpaw mechanical hypersensitivity*

Intrathecal administration of LPS leads to hyperalgesia in the hind-paw (Cahill et al., 2003, Clark et al., 2006, Clark et al., 2010a). Mice were given a priming dose of LPS (2 µg in 5 µl 0.9% sterile saline), followed by a second intrathecal injection of the same dose of LPS 24 hours later. Mechanical hypersensitivity was assessed 1- and 3 hours later. Following the final behavioural test animals were sacrificed and tissue collected. ASC^{-/-} and wildtype mice showed a significantly reduced mechanical withdrawal threshold from baseline 3 hours after LPS administration. Wildtype mice also showed a trend for reduced mechanical withdrawal threshold 1 hour after LPS administration, although this was not statistically significant ($p=0.063$). Wildtype mice exhibited a significantly lower mechanical withdrawal threshold than ASC^{-/-} mice 3 hours after LPS administration ($p=0.017$), and showed a trend for a lower mechanical withdrawal threshold 1 hour after LPS administration ($p=0.059$) (Figure 3.9).

Immunohistochemical analysis revealed no difference in the total number of microglia in the superficial lumbar dorsal horn of ASC^{-/-} mice and wildtype littermates (average 2.4 ± 0.1 cells/100 µm² and 2.4 ± 0.5 cells/100 µm² in wildtype and ASC^{-/-} animals respectively). The number of microglia expressing p-p38, indicating a state of activation, showed a trend for reduction in the superficial dorsal horns of ASC^{-/-} mice ($73.1\% \pm 8.6$ compared to $50.0\% \pm 5.7$ IBA1-positive cells expressing p-p38 in wildtype and ASC^{-/-} mice respectively, $p<0.067$) (Figure 3.10). No difference was detected in the intensity of GFAP immunoreactivity between treatment groups (data not shown).

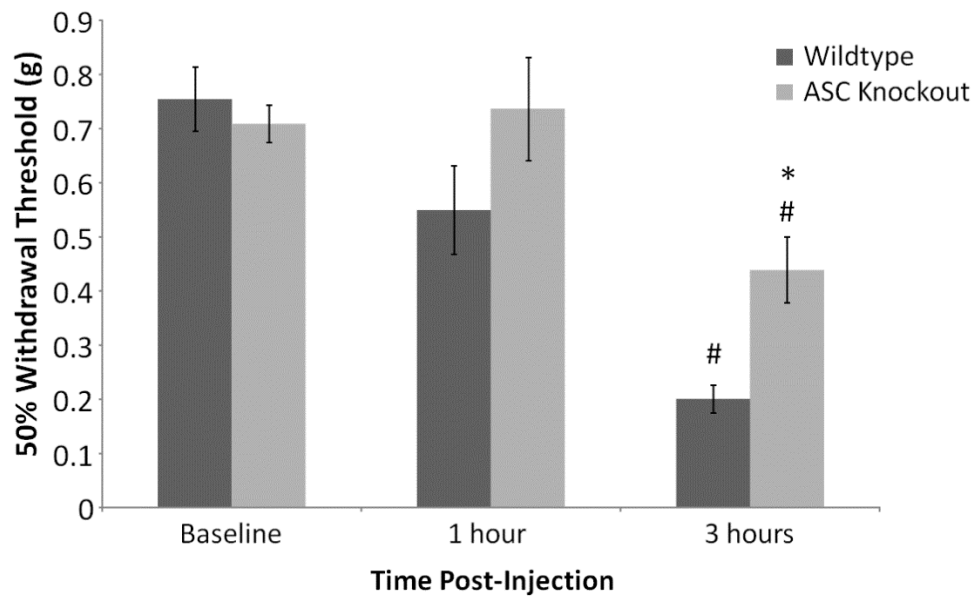


Figure 3.9: ASC deficiency results in an attenuated hindpaw mechanical hypersensitivity following intrathecal LPS administration

Intrathecal LPS produces a mechanical hyperalgesia of the hindpaw. Animals received a priming injection of LPS (2 μ g in 5 μ l 0.9% sterile saline), before receiving a second dose 24 hours later. Mechanical hypersensitivity was assessed 1- and 3 hours after the second LPS administration. Data represented as mean \pm SEM, n=8-10 mice per group. Two-way repeated measures ANOVA, followed by Dunnett's post-test, where *, p<0.05 compared to wildtype animals' responses at the same time-point, where #, p<0.05 compared to baseline responses of the same genotype. All behaviour was carried out blind to treatment.

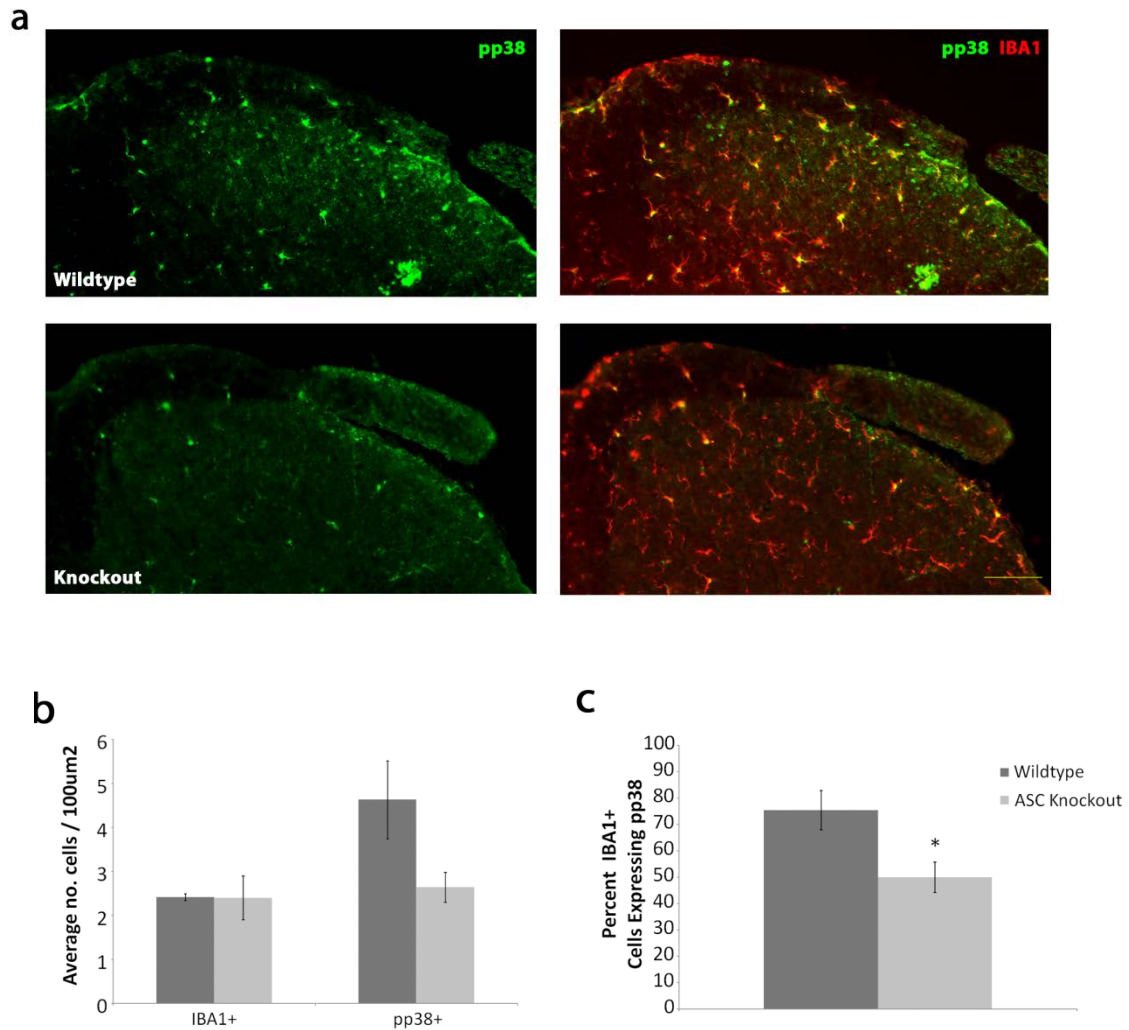


Figure 3.10: ASC^{-/-} mice show a trend for reduced microglial activation following intrathecal LPS

a: Representative images of the superficial lumbar dorsal horns of wildtype and ASC^{-/-} mice, IBA1 staining in the red channel, and p-p38 staining represented in the green channel. Scale: 100 μ m. **b:** The number of microglia in the lumbar dorsal horn did not differ significantly between genotypes. The number of p-p38 positive cells showed a trend for reduction in ASC^{-/-} mice, but was not significantly different ($p=0.068$). **c:** The number of microglia expressing p-p38 showed a trend for reduction in ASC^{-/-} mice, but was not significantly different ($p=0.067$). Data represented as mean \pm SEM, $n=3-4$ animals per group, with ≥ 3 spinal cord sections analysed per animal. Two-tailed t test.

3.6. *ASC^{-/-} mice develop comparable hypersensitivity to wildtype littermates in response to intraplantar CFA injection*

Although ASC deficiency did not affect PSNL-induced hypersensitivity, a model of neuropathic pain, ASC^{-/-} mice did show an attenuated mechanical hypersensitivity following intrathecal LPS administration, a model of central inflammatory pain. Thus it was hypothesised that the role of ASC signalling may be of greater importance in inflammatory pain. To test this hypothesis, the response of ASC^{-/-} mice to intraplantar administration of Complete Freund's Adjuvant (CFA), a model of persistent peripheral inflammatory pain, was assessed. Following CFA administration, both ASC^{-/-} mice and wildtype littermates developed a significant and lasting mechanical hypersensitivity of the ipsilateral hindpaw. There was no discernible difference between genotypes (Figure 3.11 a). Ipsilateral hindpaw withdrawal latencies to radiant heat stimulus in the Hargreaves plantar test significantly decreased at all timepoints assessed up to, and including, 10 days after CFA administration. Withdrawal latencies began to increase 10 days after CFA administration back toward baseline levels, and were not significantly different from baseline values 14 days after administration. There was no discernible difference in the withdrawal latencies of ASC^{-/-} mice and wildtype littermate mice at any timepoint tested (Figure 3.11 b). Owing to the well-characterised role of ASC in innate immune responses and inflammation, paw thickness was also assessed. Both ASC^{-/-} mice and wildtype littermates had significant paw swelling at all timepoints assessed. However, ASC^{-/-} mice showed an overall trend for reduced paw thickness following intraplantar CFA administration compared to wildtype littermates ($p=0.051$) (Figure 3.11 c).

Immunohistochemical analysis revealed no difference in the total number of microglia in the superficial ipsilateral or contralateral dorsal horns of ASC^{-/-} mice compared to wildtype littermates. Additionally no difference in microglial number was observed between superficial ipsilateral and contralateral lumbar dorsal horns. An overall difference was observed in the proportion of microglia co-expressing activation marker p-p38 between the ipsilateral and contralateral sides, though no difference between genotype was detected. No difference was observed in GFAP immunoreactivity between superficial ipsilateral and

contralateral lumbar dorsal horns of either ASC^{-/-} mice or wildtype littermates. Additionally no difference in GFAP immunoreactivity was observed between genotypes (Figure 3.12).

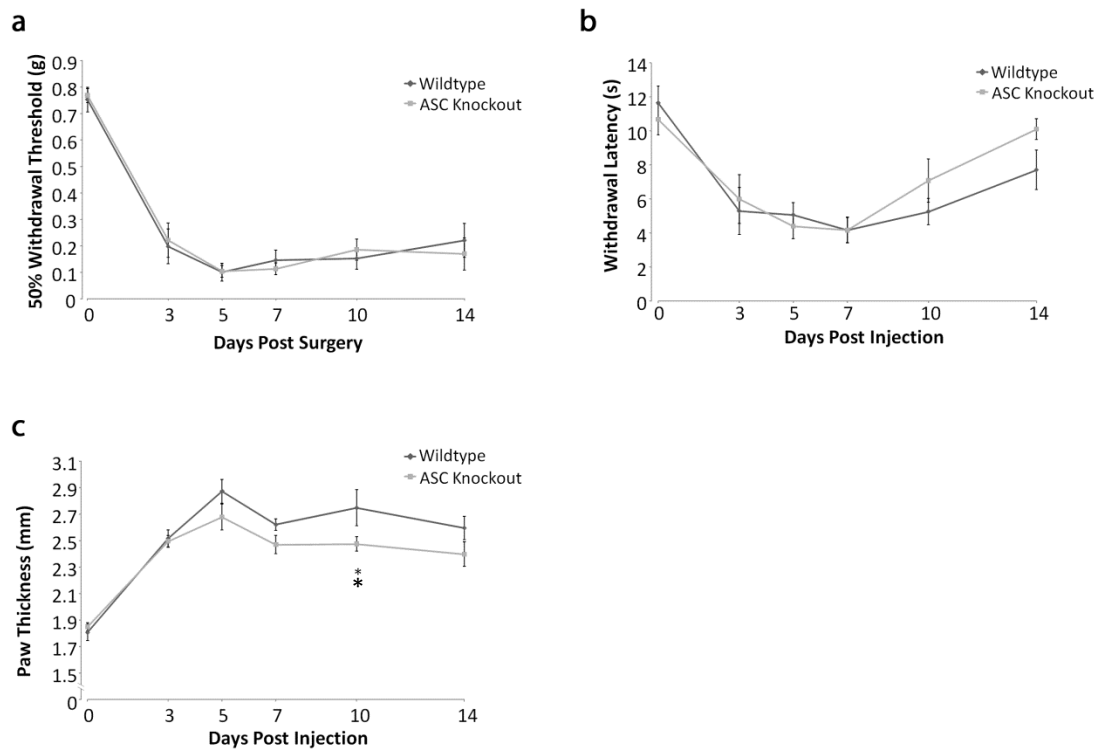


Figure 3.11: Intraplantar CFA leads to comparable behavioural hypersensitivity in ASC^{-/-} mice and wildtype littermates

a: Intraplantar CFA caused lasting mechanical hypersensitivity in the ipsilateral hindpaw in both ASC^{-/-} mice and wildtype littermates, with no discernible difference between the two genotypes. **b:** Intraplantar CFA caused a decrease in withdrawal latencies of the ipsilateral hindpaw to a radiant heat stimulus in the Hargreaves plantar test for 7 days after administration, after which time withdrawal latencies began to increase returning to close to baseline values 14 days after administration. There was no discernible difference in the withdrawal latencies of ASC^{-/-} and wildtype littermate mice at any timepoint tested. **c:** Intraplantar CFA caused a significant increase in paw thickness at all timepoints. ASC^{-/-} mice showed an overall trend for reduced paw thickness compared to wildtype littermates ($p=0.051$), with a significantly reduced paw thickness 10 days after CFA administration. Data expressed as mean \pm SEM, $n=10$ mice per group. Two-way repeated measures ANOVA, with Dunnett's post-test.

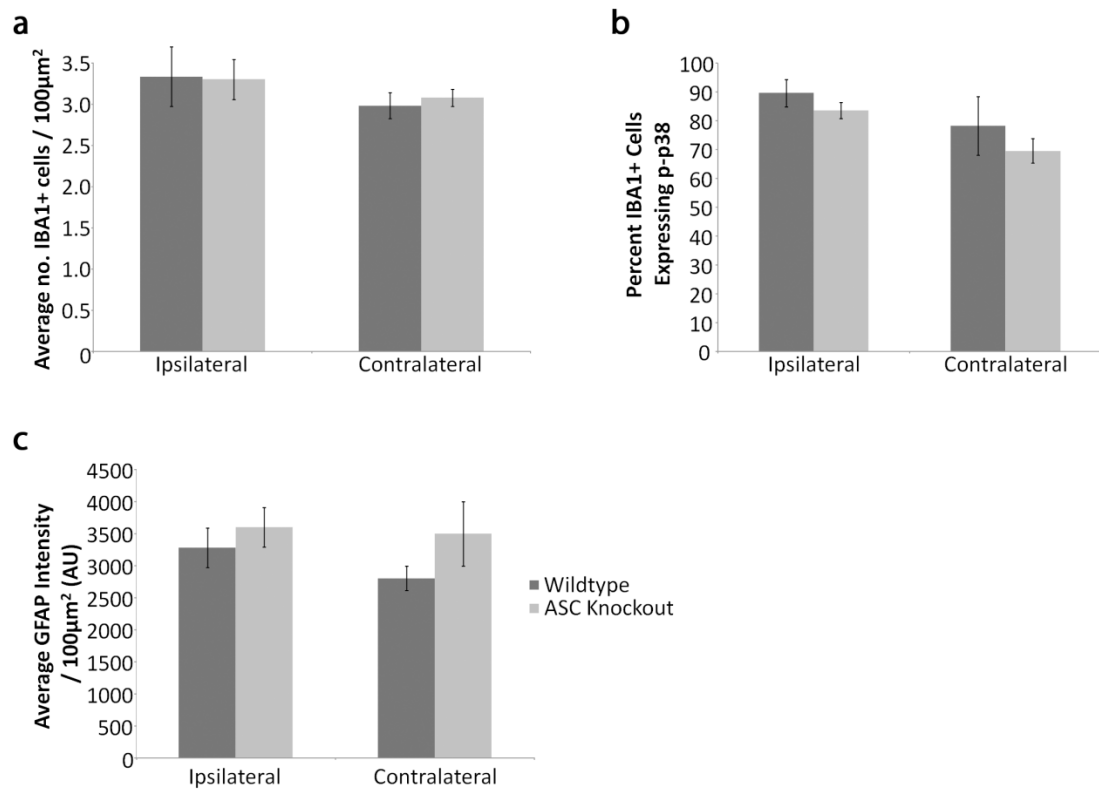


Figure 3.12: No difference in the level of microgliosis or astrocytosis in the superficial dorsal horns of ASC^{-/-} mice following intraplantar CFA administration

a: Immunostaining for microglial marker IBA1 reveals no significant difference in the number of microglia in the superficial ipsilateral and contralateral lumbar dorsal horns in either ASC^{-/-} mice or wildtype littermates. Additionally no significant difference was observed between genotypes. **b:** An overall difference was observed in the amount of p-p38 co-localisation with IBA1 in superficial ipsilateral and contralateral dorsal horns ($p=0.037$), with no significant difference observed between genotypes, **c:** Immunostaining for astrocyte marker GFAP reveals no significant difference in immunofluorescence in the superficial ipsilateral lumbar dorsal horn compared to the contralateral side in either ASC^{-/-} or wildtype littermates. No significant difference between the two genotypes was observed. Data represented as mean \pm SEM, $n=3-4$ animals per group, with ≥ 3 spinal cord sections per animal analysed. Two-way repeated measures ANOVA, with Dunnett's post-test.

4. Discussion

Data presented in Chapter 2 has suggested that caspase-1 activation contributes to the behavioural hypersensitivity observed following L5 SNT injury in rats, due to its activation of the pro-inflammatory cytokine IL-1 β and possibly also IL18. Additionally caspase-1 activity appears to play a role in the behavioural hypersensitivity observed following the intrathecal administration of LPS, an inflammatory stimulus. Thus subsequent work has focused on determining if the inflammasome, upstream of caspase-1 activation, plays a role in animal models of pain. This chapter has focused on the potential role of the adaptor protein ASC in nociceptive signalling.

4.1. The role of ASC in animal models of neuropathic pain

In the previous chapter, spinal inhibition of caspase-1 activity attenuated L5 SNT-induced hypersensitivity. This effect was not observed immediately, but instead several days after injury whether the drug was administered daily from the day of injury, or after behavioural hypersensitivity was established. Recently it has been shown that the cytokine release inhibitory drug CRID3 prevents IL-1 β release by preventing the oligomerisation of the inflammasome adaptor protein ASC (Coll and O'Neill, 2011). Because caspase-1 inhibition did not affect behavioural hypersensitivity until later timepoints, possibly due to prevention of astrocytic activation, it was decided to test the effect of ASC inhibition on established hypersensitivity in the L5 SNT model in rats. CRID3-treated animals showed a small but significant attenuation of mechanical hypersensitivity, though no change was observed in cold hypersensitivity. Given the small difference observed in mechanical hypersensitivity, this is perhaps unsurprising as the acetone test produces more variable data. It is possible that the dose tested was not high enough to inhibit ASC oligomerisation. The dose chosen was equivalent to the most effective dose shown to inhibit IL-1 β release from macrophages *in vitro* (Coll and O'Neill, 2011). Pharmacokinetic data on the drug was not available, thus it is unknown how quickly the drug was broken down. Also, YVAD-CMK is known to be cell-permeant whereas it is unknown whether or not CRID3 is similarly cell-

permeant. Another limitation of this study was the solubility of the compound. Owing to the route of administration the drug needed to be dissolved in relatively small volumes. Thus it was decided not to test a higher dose.

To gain a better understanding of the role ASC in nociceptive signalling we made use of a line of ASC^{-/-} mice. These mice were first described by Mariathasan *et al.* who showed ASC to be essential for the ATP-stimulated IL-1 β release from LPS-primed macrophages (Mariathasan *et al.*, 2004). ASC^{-/-} mice have since been studied extensively, with the focus mainly on its role in innate immunity. However this is the first time these mice have been employed in the study of nociception. Data presented here indicate that acute nociceptive signalling is unaltered in ASC^{-/-} mice, as indicated by comparable responses to hot and cold plate tests, radiant heat stimulus (Hargreaves test), and to punctate mechanical stimuli (von Frey test). Locomotor function is also unaffected by gene deletion, as assessed by the RotaRod test.

The effect of ASC gene deletion on behavioural hypersensitivity in an animal model of neuropathic pain was assessed first. In previous studies, using rats, the L5 SNT model was employed. However this injury is technically more challenging to carry out in mice, and so the PSNL model was used. Preliminary experiments showed that PSNL injury in mice caused mechanical hypersensitivity and microgliosis similar to that observed in SNT-injured rats. PSNL injury in ASC^{-/-} mice caused a comparable mechanical and thermal hypersensitivity to that observed in wildtype littermates. Immunohistochemical analysis showed a comparable microgliosis and astrocytosis between genotypes, which is perhaps unsurprising given the lack of behavioural difference. If ASC oligomerisation is directly upstream and obligatory for caspase-1 activation, then this data is at variance with that of the previous chapter where caspase-1 inhibition attenuated hypersensitivity in the L5 SNT model of neuropathic pain. This difference could be, at least in part, due to a species difference. This idea is possibly supported by the finding that CRID3 attenuated mechanical hypersensitivity in the rat after L5 SNT, although the effect was small. There are several factors which may contribute to this apparent discrepancy, which will be outlined in the following sections.

4.1.1. Differences in animal models of neuropathic pain

Another fundamental difference between these studies is the model used. Behavioural differences in animal models of neuropathic pain induced by peripheral nerve injury have been previously compared (Kim et al., 1997, Dowdall et al., 2005), with the conclusion that, although each model produced behavioural hypersensitivity in a range of different tests, there were differences in the magnitude and timecourse of hypersensitivity across several sensory modalities. In addition to differences in behavioural outcomes, there has been some attempt to look at differences in molecular changes in these models (Lee et al., 1998, Scholz et al., 2008, Casals-Diaz et al., 2009, Maratou et al., 2009, Noma et al., 2011). For example extensive sympathetic sprouting in the injured DRG of SNT-injured rats was observed 1 week after injury, and gradually declined over several weeks. However no sympathetic sprouting was detected in the DRGs of animals following PSNL or CCI injury until 20 weeks after injury. Moreover the extent of sprouting was considerably less than that observed in SNT-injured rats (Lee et al., 1998). Differences have also been detected in spinal cord plasticity following CCI, SNI, and crush injuries as assessed by the extent of changes in the pattern of SP- and IB4-immunoreactivity in the spinal cord dorsal horn (Casals-Diaz et al., 2009). Changes in gene expression following L5 SNT injury have also recently been explored, and compared to a HIV-associated model of neuropathic pain, which does not involve peripheral nerve injury. The study showed that although there was some convergence in gene changes detected between the two models, ultimately there were many more genes which were dysregulated which differed between the two models (Maratou et al., 2009). Thus it is clear that although there are several nerve-injury induced models of pain resulting in pain-related hypersensitivity, there may be underlying differences in the pathophysiology between models. This is not to say that there are not commonalities between models, however. Scholz *et al.* looked at potential differences in the timecourse and extent of microgliosis in CCI, SNI, and SNT models of neuropathic pain in rats. The group reported a similar extent of microgliosis across a similar timecourse in all models tested (Scholz et al., 2008). Data presented in previous chapters indicate that the inflammasome is expressed in microglia, and so it might be expected that changes in inflammasome signalling across these models is

similar. However it is important to consider that microglial changes in animal models of pain occur as a result of neuronal-glial crosstalk. Thus differences in neuronal changes across models could, potentially, lead to differences in the degree of microglial activation and intracellular signalling.

Another consideration in the apparent conflicting results of these studies is that CRID3, which was only minimally effective, might not have been acting solely at the level of ASC oligomerisation and may have been efficacious via some unknown, off-target, effect. Additionally it is possible that the antinociceptive effect of Ac-YVAD-CMK may have been due not only to caspase-1 inhibition, but also some off-target effect of other pro-inflammatory caspases. It has been shown that Ac-YVAD-CMK can inhibit caspase-4 and caspase-5, though with a considerably higher K_i (Rabuffetti et al., 2000).

4.1.2. Redundancy in inflammasome interactions

A further consideration is the possibility of compensation of the adaptor protein ASC in ASC^{-/-} mice. ASC participates in the assembly of the inflammasome complex via homotypic protein-protein interactions. There are several other proteins already identified which possess the PYRIN domain. Many of these have been identified as endogenous inhibitors of inflammasome assembly, for example POP1, POP2, and PYNOD (Wang et al., 2004a, Stehlik and Dorfleutner, 2007). However there are also PYRIN domain-possessing proteins which have been suggested to act as adaptors for inflammasome complexes. The first description of an inflammasome complex, the NLRP1 inflammasome, described two adaptors; ASC and Cardinal (Martinon et al., 2002). Cardinal has been shown to interact with the NLRP3 inflammasome in macrophages from patients with Muckle-Wells syndrome (Agostini et al., 2004). However it is thought that Cardinal is not expressed in the mouse genome, so a role for Cardinal in this instance is ruled out (Lamkanfi et al., 2007, Martinon and Tschopp, 2007). It has been suggested that the murine NLRP1 protein, which contains a FIIND (function to find) domain, a CARD domain, but lacks a PYRIN domain, may serve a similar function to human Cardinal, owing to their structural similarity (Martinon and Tschopp, 2007). It is possible that NLRP1

signalling, not requiring an adaptor protein, may activate caspase-1 as well as, or in compensation for, ASC-induced caspase-1 activation. The NLRC4 (previously named IPAF) inflammasome protein also contains a CARD domain meaning it is also capable of activating caspase-1 directly, although by some unknown mechanism, interaction with ASC (NLRC4 does not contain a PYRIN domain) enhances its caspase-1 activating function (Mariathasan et al., 2004). Thus it is possible that another inflammasome complex, which does not require an adaptor protein, is either responsible for caspase-1 activation in animal models of neuropathic pain, or alternatively may be activated in the case of ASC^{-/-} mice in a compensatory manner.

RIP2 (receptor-interacting protein 2, also known as CARDIAK and RICK) is a recently described caspase-1-activating protein. It is a serine/threonine kinase with a CARD domain and has been shown to interact with several CARD-containing proteins including caspase-1, leading to its activation. RIP2 activation occurs downstream of TLR2, 3, and 4 receptor signalling (Kobayashi et al., 2002). When first identified RIP2 was shown to interact directly with the CARD domain of caspase-1, as well as with the TNF receptor adaptor molecules TRAF-1 and TRAF-2. Furthermore overexpression of RIP2 induced activation of NF κ B and JNK (Thome et al., 1998). However data from Kobayashi *et al.* suggests that RIP2 is not critically involved in TNF-receptor signalling since TNF- α stimulation of RIP2-null embryonic fibroblasts leads to IL-6 production normally. In the same report the authors found that RIP2-null mice showed deficits in IL-1 β and IL-18 signalling, both of which share similarities with TLR signalling pathways (Kobayashi et al., 2002). Thus it is possible that RIP2-mediated caspase-1 activation, and not activation via the inflammasome complex, contributes to behavioural hypersensitivity in animal models of neuropathic pain. This might explain why caspase-1 inhibition attenuated behavioural hypersensitivity in L5 SNT-injured rats, but ASC deficiency did not affect PSNL injury-induced behavioural hypersensitivity.

4.2. The role of ASC in a model of central inflammatory pain

The response of ASC^{-/-} mice to LPS-induced hypersensitivity was also

assessed. This experimental paradigm was initially chosen as a model for inflammation for several reasons. Firstly it is a model of central inflammation, without any complicating contributory factors from the periphery. Secondly, it relates closely to the *in vitro* experiments carried out in Chapter 1. ASC^{-/-} mice showed an attenuated mechanical hypersensitivity in response to LPS compared to wildtype littermates. Immunohistochemical analysis showed a trend toward reduced microglial activation, as indicated by reduced colocalisation with activation marker p-p38. This was not statistically significant, and greater insight would have been provided with a greater number for immunohistochemical analysis. Caspase-1 inhibition similarly led to an attenuation of LPS-induced hindpaw sensitivity and also a reduction in microglial activation. It is therefore possible that the attenuation of mechanical hypersensitivity in ASC^{-/-} mice was also mediated by a reduction in microglial activation. LPS is a commonly used priming stimulus in investigations of inflammasome activation and signalling *in vitro*, and stimulation has been shown to lead to dose-dependent increases in *Nlrp3* mRNA (O'Connor et al., 2003, Kahlenberg et al., 2005, Bauernfeind et al., 2009). In this model a priming LPS stimulus is used, which potentially results in the upregulation of NLRP3. Subsequent activation of the NLRP3 protein would result in inflammasome assembly, which would require an adaptor protein. However, 3 hours after LPS administration, although significantly attenuated compared to wildtype littermates, ASC^{-/-} mice displayed significant mechanical hypersensitivity compared to baseline responses. Thus although ASC contributes to LPS-induced hypersensitivity it is not indispensable for this process.

LPS administration to *ex vivo* dorsal horn slices has been shown to lead to the release of caspase-1 and ASC, as well as IL-1 β (Clark et al., 2006). This, in line with *in vitro* data, suggests that the inflammasome is activated following intrathecal LPS. Data in Chapter 1 suggest the inflammasome is expressed in microglia, also known to express TLR4 receptors, the receptor through which LPS acts, and the activation of which has been shown to contribute to behavioural hypersensitivity in animal models of neuropathic pain (Tanga et al., 2005, Bettoni et al., 2008). Data here suggest that ASC, and so possibly also

the inflammasome, contributes to LPS-induced hypersensitivity, but not exclusively. It has been shown that microglia from NLRP3^{-/-} and ASC^{-/-} mice stimulated with intact *S. aureus* secrete significantly reduced levels of IL-1 β . However, the response is not completely ablated. Thus there are other, independent, cellular mechanisms capable of bringing about IL-1 β maturation and release in response to this inflammatory stimulus. Interestingly IL-18 maturation and release was unaffected in response to this stimulus suggesting that, under certain conditions, IL-1 β and IL-18 maturation and release are differentially regulated (Hanamsagar et al., 2011). Thus it is possible that the mechanical hypersensitivity of ASC^{-/-} mice following intrathecal LPS administration seen in this study was due to some residual IL-1 β release, or IL-18, by some other ASC-independent mechanism. Intrathecal administration of IL-18 has been shown to elicit mechanical hypersensitivity in rats (Miyoshi et al., 2008). Differences in mature IL-18 expression in spinal cord lysates was investigated, but this proved to be technically challenging and it was not possible to detect expression of the mature cytokine in either genotype. RIP2 has been shown to be upregulated in macrophages following LPS stimulation. Although RIP2 is known to be activated downstream of TLR4 signalling, and to interact with caspase-1, macrophages from RIP2^{-/-} mice did not show a deficit in IL-1 β production when stimulated with LPS. The authors suggested that this could mean that RIP2 is not involved in caspase-1 activation downstream of TLRs, or alternatively RIP2-mediated caspase-1 activation is not the dominant means of activation in this situation and is a redundant mechanism (Kobayashi et al., 2002). Potentially RIP2-mediated caspase-1 activation is responsible for the mechanical hypersensitivity observed in ASC^{-/-} mice in this study. This could explain why rats treated with caspase-1 inhibitor Ac-YVAD-CMK did not display mechanical hypersensitivity following intrathecal LPS administration.

Alternatively the residual mechanical hypersensitivity of ASC^{-/-} mice following intrathecal LPS administration may be due to separate signalling mechanisms, independent of IL-1 β and IL-18. Clark *et al.* have shown that the protease Cathepsin S is released from activated microglia in the dorsal horn following LPS application to *ex vivo* spinal cord slices. Secreted Cathepsin S cleaves

neuronal membrane-bound fractalkine which acts on glial CX3CR1 receptors, leading to further glial activation (Clark et al., 2009). Additionally intrathecal LPS administration has been shown to increase expression of TNF- α in microglia (Shen et al., 2009). These factors, and others, could be responsible for nociceptive signalling following intrathecal LPS administration.

4.3. The role of ASC in a model of peripheral inflammatory pain

That ASC-/- mice showed a difference in behaviour following intrathecal LPS administration, but not after PSNL injury, led to the hypothesis that ASC, and perhaps inflammasome signalling, may have a greater contribution to inflammatory pain mechanisms than neuropathic pain. To test this, ASC-/- mice, and wildtype littermates, were given intraplantar CFA, a well-documented model of persistent peripheral inflammatory pain, and subsequent behaviour was assessed. The level of inflammation of the injected hindpaw of ASC-/- mice, as assessed by paw thickness measurements, was found to be overall significantly less than that of wildtype littermates. The inflammasome is known to be important in the innate immune response, so it is perhaps not surprising that the level of peripheral inflammation is reduced in ASC-/- mice. However, despite a reduced level of inflammation of the hindpaw, mechanical and thermal hypersensitivity of ASC-/- mice did not differ from that of wildtype littermates, suggesting that inflammasome signalling does not contribute to CFA-induced hypersensitivity. In a study investigating inflammasome responses to adjuvants commonly used in vaccines it was found that LPS-primed macrophages from wildtype, NLRP3-/-, and ASC-/- mice showed comparable IL-1 β release when stimulated with CFA (Eisenbarth et al., 2008). However this was just one cell type, in an *in vitro* assay. CFA is known to elicit an inflammatory reaction which sensitises peripheral nociceptors and leads to central sensitisation (Okun et al., 2011). Thus although it is thought that CFA does not directly activate the inflammasome in the periphery, the experimental question in this study was whether or not ASC-deficiency affected the development of behavioural hypersensitivity, driven by inflammation-mediated peripheral and central sensitisation. In the present study it was shown that ASC-deficiency did not prevent CFA-induced behavioural hypersensitivity.

There is conflicting data on the role of spinal glia in the CFA model of inflammatory pain. In one report spinal microgliosis and astrocytosis were reported in the rat following hindpaw administration of CFA, although whether this was uni- or bilateral is unclear (Raghavendra et al., 2004). In the present study no difference was detected in microglial number in the superficial lumbar dorsal horn between the ipsilateral and contralateral sides. A better understanding of these data would have been obtained if the study had included sham-injected mice; if a bilateral change in microglial number had occurred this would have been detected. Using microglial morphology as a marker of activation, Lin *et al.* reported no change in spinal microglial activation following intraplantar CFA administration, and no change in microglial number (Lin et al., 2007). Another group, however, reported limited change in microglial number, with some change in morphology (Lee et al., 2010b). Subjectively, in this study, it was felt that microglia in the ipsilateral superficial dorsal horn had larger soma. However this was not quantified. Instead the level of microglial activation was assessed by the extent of colocalisation with activation marker p-p38. An overall statistical difference was detected in the level of p-p38 colocalisation with IBA1 between the ipsilateral and contralateral superficial lumbar dorsal horns. No difference was detected between genotypes, perhaps unsurprisingly given that no behavioural difference was observed. Additionally no difference in GFAP immunoreactivity was observed.

4.4. Conclusions

Data presented in this chapter indicate that ASC plays an important role in LPS-induced hypersensitivity. This is in line with *in vitro* data presented in Chapter 1, as well as various published reports, where it was shown that ATP-stimulation of LPS-primed primary microglia and macrophages led to caspase-1 activation and IL-1 β release. Published data indicates that this is inflammasome-dependent since genetic ablation of NLRP3 and ASC leads to either a substantial reduction or abolition of IL-1 β release in response to LPS depending on the cell type and experimental paradigm (Mariathasan et al.,

2004, Sutterwala et al., 2006, Halle et al., 2008, Hanamsagar et al., 2011). ASC does not appear to have a more general role in inflammatory pain, however, as assessed by the lack of behavioural differences in ASC^{-/-} mice and wildtype littermates in the CFA model of inflammatory pain. However, it was hypothesised that the inflammasome played a role in nociceptive signalling through IL-1 β , and possibly IL-18, release from microglia and macrophages. In the present study, although there was a small difference in ipsilateral spinal microglial activation, there was no change in the number of microglia ipsilaterally. Differences in macrophage activation were not investigated. Thus this may not have been an ideal model of inflammation to investigate the role of the inflammasome. Following PSNL an ipsilateral spinal microgliosis was observed. However ASC^{-/-} mice showed comparable behavioural hypersensitivity to wildtype littermates. This may reflect a compensation of other inflammasome proteins not requiring an adaptor protein, for example NLRP1 or NLRC4, or this may reflect compensation of another, as yet unidentified, adaptor protein. Alternatively this may be due to an inflammasome-independent activation of caspase-1 for example by RIP2. In conclusion ASC contributes to LPS-induced inflammatory pain, but does not contribute to CFA-induced inflammatory pain or PSNL injury-induced neuropathic pain.

Chapter 4: The Central Inflammasome Scaffold Protein NLRP3 Does Not Contribute to Behavioural Hypersensitivity in an Animal Model of Neuropathic and Central Inflammatory Pain

1. Introduction

Data presented thus far shows that caspase-1 signalling contributes to mechanical and cold hypersensitivity in the L5 SNT model of neuropathic pain, and following intrathecal LPS administration as a model of central inflammation. The activation of caspase-1 in neuropathic pain appears to be inflammasome-independent since ASC^{-/-} mice develop mechanical and thermal hypersensitivity normally in the PSNL injury model of neuropathic pain. However ASC^{-/-} mice did show a significantly attenuated hindpaw mechanical hypersensitivity following intrathecal LPS administration. The current chapter explores the role of NLRP3 in animal models of pain by looking at the effect of genetic ablation of NLRP3 expression.

The NLRP3 inflammasome has been extensively studied in the field of immunology, specifically exploring its role in innate immunity. Several pathogens, including malarial hemozoin, muramyl dipeptide, lipopolysaccharide, *sendai virus*, and *influenza virus* have been shown to elicit an inflammatory response via the activation of the NLRP3 inflammasome (Mariathasan et al., 2006, Martinon et al., 2007, Dostert et al., 2009). Additionally the NLRP3 inflammasome has been shown to be activated by a variety of endogenous factors indicating stress or injury, often referred to as danger-associated molecular patterns (DAMPs). These include ATP, uric acid, and β -amyloid (Mariathasan et al., 2006, Martinon et al., 2006, Ogura et al., 2006, Halle et al., 2008, Gasse et al., 2009). In this respect NLRP3 is unique among the inflammasomes; the range of activators of other inflammasome complexes is more limited, and generally limited to exogenous, pathogenic, activators (Martinon and Tschopp, 2007, Schroder and Tschopp, 2010).

There are now several published reports indicating a role for NLRP3 in a range of pathological conditions including type 2 diabetes, atherosclerosis, Alzheimer's disease, and obesity (Halle et al., 2008, Zhou et al., 2010, Leemans et al., 2011, Collino et al., 2012). Given an already established role of IL-1 β and IL-18 in several animal models of pain (Wolf et al., 2006, Miyoshi et al., 2008, Li et al., 2009, Ren and Torres, 2009), as well as data presented in Chapter 2 indicating a role for caspase-1 in nociceptive signalling, the role of NLRP3, and by implication the NLRP3 inflammasome, was assessed in an animal model of neuropathic and central inflammatory pain. Although inferences about the role of the inflammasome in nociceptive signalling can be drawn from studies presented in Chapter 3, looking at the effect of inhibition and deletion of the adaptor protein ASC, investigating the effect of NLRP3 deletion provides a more complete understanding of the role of the inflammasome in nociception. In particular it is important to investigate all levels of the inflammasome pathway since ASC has, in some experimental paradigms, been shown to act independently of the inflammasome (Ippagunta et al., 2010, Shaw et al., 2010, Ellebedy et al., 2011), although there are conflicting reports to these (Rosengren et al., 2005, Gris et al., 2010).

2. Methods

2.1. *Animals and surgery*

Adult (10 – 12 weeks) male and female NLRP3-/- (sourced from Genentech), bred on a C57Bl/6 background, and age- and sex-matched C57Bl/6 mice (Harlan, UK) were used in accordance with UK Home Office regulations (Animals Scientific Procedures Act 1986).

Genotyping: A tissue sample of mouse pups (p10 – p14) was obtained by ear punch (Harvard Apparatus, UK), which was also used for identification purposes. The tissue incubated with 30 µl lysis buffer (consisting of 67mM Tris (Sigma), 16mM (NH₄)₂SO₄ (Sigma), 6.7mM MgCl₂ (Sigma), 0.2%w/v β-mercaptoethanol (Sigma), 0.4 mg/ml Proteinase K (VWR)) at 55°C for 1 hour, followed by 10 minutes at 95°C. Samples were briefly vortexed, then centrifuged, with the resultant supernatant containing the DNA used for PCR. Genotype was determined by a polymerase chain reaction (PCR). The master mix solution consisted of 1.5 mM MgCl₂ (Promega), 20% v/v 5x Reaction Buffer (Promega) 0.2 mM dNTP (Promega), 50 units/ml Taq DNA polymerase (Promega), 0.1 µM primers x 3 (Sigma), 0.5 µl DNA sample.

Primer sequences:

Cryopyrin-F (forward): CCA GCC AGA GTG GAG TGA GTA AAC

Cryopyrin-R (reverse): CAG TCA CCT GCG AGTGAG TGA AGA C

Cryopyrin-Neo (forward): CCG GTG GAT GTG GAA TGT GTG C

Each reaction consisted of 0.5 µl DNA, and 24.5 µl master mix. The PCR reaction was performed in TC412 Flexigene thermal cycle (Techne, UK). The conditions for the PCR reaction were as follows: denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds, and polymerisation at 72°C for 1 minute. This was followed by a final extension step at 72°C for 10 minutes. The expected size of the products were 665bp for wildtype, 318bp for knockout, and 665 and 318bp for heterozygotes.

Visualisation: To visualise the PCR products, gel electrophoresis of the samples was performed, using a 2% agarose gel containing 100 µg/ml

ethidium bromide (Sigma) for visualisation. Gels were viewed using GeneGenius system (Syngene), and imaged using GeneSnap (Syngene).

Partial sciatic nerve ligation: Under isoflurane anaesthesia, mice the left sciatic nerve was exposed at mid-thigh level. A 5-0 silk suture was used to tightly ligate approximately a third to half of the nerve. The wound was closed using two wound clips. No post-surgical analgesia was given in order to avoid potential modulation of the nociceptive and immune system of the animals. The general health of the animals was monitored daily after the surgery.

Drugs and delivery: LPS was administered as 2 µg dissolved in 5 µl sterile saline. Awake mice were lightly restrained and a 10 µl Hamilton syringe was inserted between the L5 and L6 vertebrae, where the spinal cord consists mainly of spinal roots. The accuracy of placement was ensured through the observation of a tail flick. The needle was slowly withdrawn to ensure no loss of drug. Injections were kindly carried out by Mr. Clive Gentry.

As described in Chapter 3, animals were either sacrificed by terminal anaesthesia and transcardially perfused with either 4% paraformaldehyde plus 1.5% picric acid for histology, or saline alone for Western blot analysis. Tissue used for histology: Lumbar spinal cords were excised and post-fixed overnight. Tissue was cryoprotected in 20% sucrose in 0.1M Phosphate Buffer (PB) for 4-7 days before OCT embedding. Tissue used for Western blot analysis: Lumbar spinal cords were snap-frozen in liquid nitrogen and stored at -80°C.

2.2. Behavioural testing

Animals were tested in a temperature stable room during the light period of their day/night cycle, at the same time every day. Animals were placed in a polymethyl methacrylate box with a wire grid bottom and allowed to habituate for a period of 15-30 minutes before testing. Animals were trained once for each behavioural test, and then two baseline tests were performed on different days.

Assessment of mechanical withdrawal thresholds: Mechanical withdrawal thresholds were assessed using calibrated von Frey hairs (Ugo Basile) according to the “up-down” method (Chaplan et al., 1994), as described in Chapter 1. The 50% withdrawal threshold was then calculated using the method described by Dixon (Dixon, 1980).

Assessment of cold hypersensitivity – acetone test: For measurement of cold hypersensitivity, a drop of acetone was applied to the plantar surface of the rat hindpaw, as described in Chapter 1. 3 tests were carried out, at least 10 minutes apart from each other, and an average response was recorded.

Assessment of heat thresholds - Hargreaves test: The noxious heat threshold of the hind paw was determined using Hargreaves Plantar Test (Hargreaves et al., 1988). Animals were placed in acrylic cubicles (8 x 5 x 10 cm) atop a uniform glass surface, and allowed to habituate before testing. An infrared light source was directed to the plantar surface of the hind paw, and the latency to withdraw was recorded. To prevent tissue injury, the maximum stimulus duration was 20 seconds. Three responses were recorded for each hind paw, and an average response for each taken.

Hot / cold plate test: Response to continued noxious thermal stimulation of the paws was assessed using an Incremental Hot / Cold Plate (IITC Life Sciences, Woodland Hills, CA, USA), set at 4, 50, and $52 \pm 0.1^{\circ}\text{C}$, with latency to lick or flinch the hindpaws recorded. To avoid tissue injury animals were removed after 20 seconds if no response was observed. Three responses were recorded, and an average response taken.

Locomotor function: Locomotor function was assessed using an accelerating RotaRod (Ugo Baile), set to accelerate from 2 to 40 rpm over a period of 560 seconds. Latency to fall was recorded. Three responses were recorded, and an average response taken.

All behavioural testing was carried out blind to treatment, or genotype.

2.3. Western blot

Tissues were homogenised in the same way as described in Chapter 1. Protein concentration of all samples was determined using a BCA Protein

Assay kit (Thermo Scientific). Lysates were normalised to 35 µg protein per sample. Proteins (35 µg lysate per well) were separated using SDS-PAGE and transferred to 0.2 µm pore size PVDF membranes (Millipore). Membranes were blocked in 10% skimmed milk in PBS-T for 45 minutes before overnight incubation with primary antibody at 4°C as already described in Chapter 1. Species appropriate HRP-conjugated secondary antibody (1:10,000, Amersham) was applied the subsequent day for 1 hour at room temperature. Membranes were visualised using ECL-prime reagent for 5 min (GE Healthcare) before detection by autoradiography. For visualisation of protein loading control, the membranes were stripped using a β-mecaptoethanol buffer as described in Chapter 1, before incubation with primary antibody.

Antibodies used: mouse anti-β-actin (1:15,000, Sigma), donkey anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:20,000, GE Healthcare).

Blots were analysed by determining mean intensity of each band using a fixed box size for all. The background intensity for each blot was then subtracted from all values obtained. All quantifications were then normalised against β-actin loading control. All blots were then compared to the mean band intensity for control-treated samples or naive animals. Analysis was carried out using Quantity One 1-D Analysis Software version 4.6.9 (Biorad).

2.4. Histology

Lumbar spinal cords were cryostat cut in 20 µm sections and thaw-mounted onto glass slides. When slides were fully dry (at least 1 hour after tissue mounting), they were stored in cryoprotectant (30% ethylene glycol, 30% glycerol, 40% PBS) at -20°C until required.

Immunohistochemistry was carried out according to the methods described in Chapter 1. Primary antibodies used: Rabbit anti-GFAP (1:1000, DAKO), rabbit anti-IBA1 (1:1000, WAKO), rabbit anti-p-p38 (1:100, Cell Signalling). Tyramide signal amplification was used to visualise p-p38. Secondary antibodies: anti-

mouse or anti-rabbit Alexa Fluor 488 (1:1000, Stratech), anti-mouse or anti-rabbit Alexa Fluor 546 (1:1000, Stratech), ExtrAvidin FITC (1:200, Sigma). Specificity of staining was determined by omission of the appropriate primary antibody as a negative control. Slides were mounted on coverslips using Vectashield mounting medium with DAPI (Vectorlabs) and visualised using a Zeiss Axioplan 2 fluorescent microscope.

Quantification:

Cell counts / colocalisation: 3 100 μm^2 boxes were drawn over the superficial L5 dorsal horn, ipsilateral and contralateral to the injury. Cells which were associated with a nuclear stain, within the confines of the box, were counted. An average count from the 3 boxes was taken. At least 3 sections per animal were analysed. Counts were performed manually. Analysis was carried out blind to treatment group / genotype.

GFAP intensity: All GFAP images had been taken on the same day, using the same exposure. As for the cell counts, 3 100 μm^2 boxes were drawn over the superficial L5 dorsal horn, ipsilateral and contralateral to the injury. A 20 μm^2 box was drawn in the white matter, and was used to generate a background intensity value. The intensity of the 3 boxes, minus the background intensity value, was averaged. At least 3 sections per animal were analysed. Analysis was carried out using AxioVision LE version 4 software.

2.5. Statistical analysis

All data are represented as mean \pm SEM where appropriate. Western blot and histology data were analysed using one-way ANOVA followed by Dunnett's post-test. Behavioural data were analysed using two-way repeated measures ANOVA followed by Dunnett's post-test. Where data were not normally distributed, ANOVA tests were performed on ranked data. All analysis was carried out using the statistical package SigmaPlot for Windows, version 12.

3. Results

3.1. Assessment of acute nociceptive behaviour in NLRP3^{-/-} mice

The role of the central scaffold inflammasome protein NLRP3 in animal models of pain was investigated using a strain of mice deficient in NLRP3 expression. The generation of these mice has been described previously, and is illustrated in Figure 4.1. Owing to initial breeding difficulties, along with time constraints, all experiments carried out with NLRP3^{-/-} mice used age- and sex-matched C57Bl/6 mice as wildtype controls (termed “wildtype” in figure legends) as opposed to wildtype littermates. Western blot of spinal cord lysates from NLRP3^{-/-} mice and C57Bl/6 mice generated several bands. Despite this, a difference was detected between the two genotypes with a band of approximately 50kDa in size produced by C57Bl/6 spinal cord lysates, absent from blot of NLRP3^{-/-} spinal cord lysates. Additionally a band of approximately 25kDa in size was detected in NLRP3^{-/-} spinal cord lysates, which was absent from spinal cord lysates from C57Bl/6 mice. This may be a truncated, non-functional, form of the NLRP3 protein, since exons 4 and 5 of the NLRP3 gene remain (Mariathasan et al., 2006). However it should be noted that the band detected in the spinal cord lysates of C57Bl/6 mice is smaller than that which would be expected, of around 70 - 80kDa dependent on the number of LRR regions (see Chapter 1, (Kummer et al., 2007)).

The effect of NLRP3 gene deletion on sensory processing pathways has not previously been investigated. Thus the behaviour of NLRP3^{-/-} mice and C57Bl/6 wildtype mice was assessed in a range of tests to determine if there was any difference in acute pain processing in response to peripherally-applied mechanical and thermal stimuli. NLRP3^{-/-} mice showed a significantly increased withdrawal latency in the coldplate test, set at 4°, and in the hotplate test set at 50°C, but not 52°C. However, no difference was observed between NLRP3^{-/-} mice and C57Bl/6 mice in withdrawal latency to a gradual radiant heating of the plantar surface in the Hargreaves test. NLRP3^{-/-} mice also exhibited equivalent withdrawal thresholds to von Frey mechanical stimuli. No

deficit in motor function was observed, as assessed by the RotaRod test (Figure 4.2).

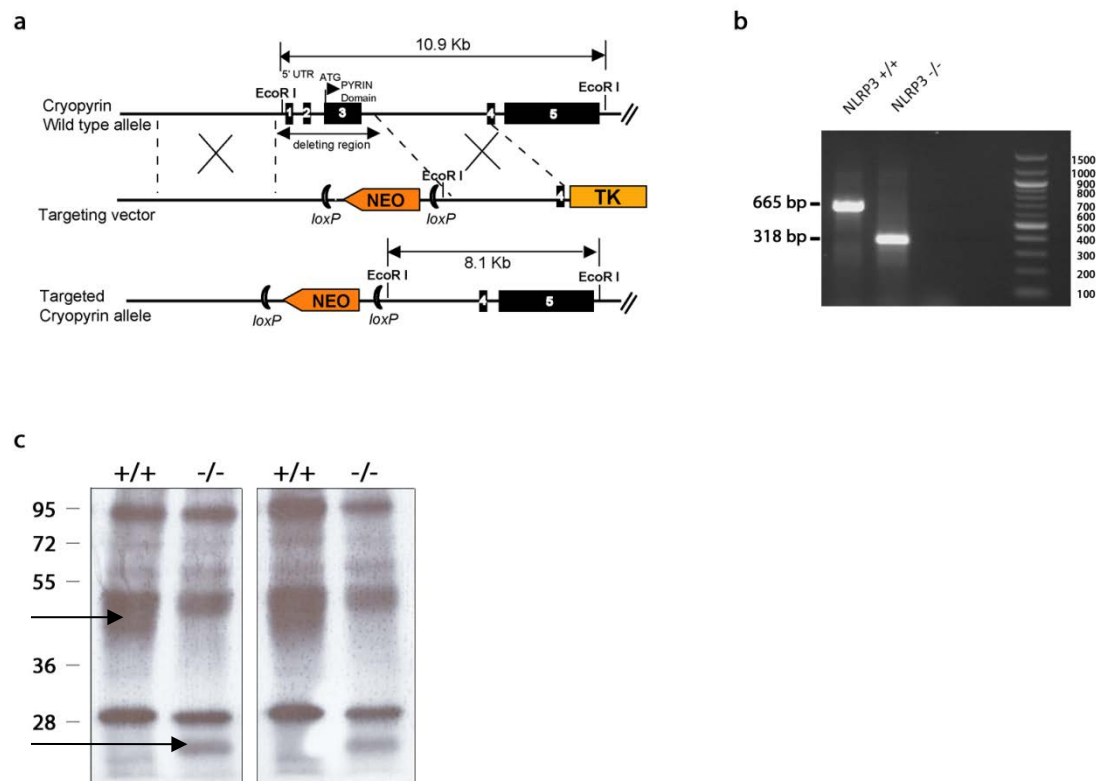


Figure 4.1: Targeted disruption of the murine NLRP3 gene

a: Schematic demonstrating the targeted disruption of the murine NLRP3 gene. Exons (closed black boxes) 1, 2, and 3 encompassing a 5' untranslated region (5' UTR), start codon, and the PYRIN domain of the protein were replaced with a neomycin resistance cassette. Exons 4 and 5 remain. X up- and downstream of this cassette indicate homology arms. TK indicates a thymidine kinase negative selection marker, outside the region of sequence similarity between the wildtype allele and targeting vector. Diagram adapted from (Mariathasan et al., 2006). **b:** Representative agarose gel of PCR products from genotyping, with wildtype mice yielding a 665bp product, and NLRP3^{-/-} mice yielding a 318bp product. **c:** Western blot for NLRP3 of spinal cord lysates from C57Bl/6 mice and NLRP3^{-/-} mice yields several bands. Arrows indicates a band of approximately 50kDa present in C57Bl/6 mice spinal cord lysates, absent from spinal cord lysates of NLRP3^{-/-} mice, and a band of approximately 25kDa present in NLRP3^{-/-} mice spinal cord lysates, but absent from C57Bl/6 mice spinal cord lysates.

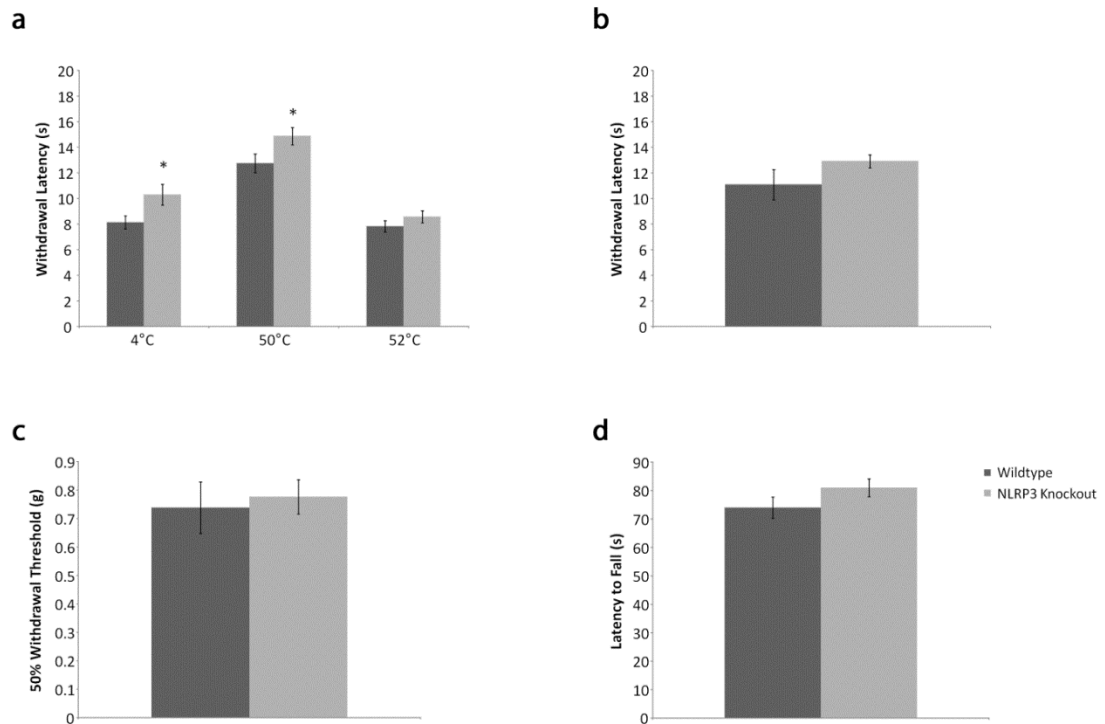


Figure 4.2: NLRP3^{-/-} mice do not display normal responses to acute thermal and mechanical noxious stimuli

The withdrawal thresholds of naïve NLRP3^{-/-} and C57Bl/6 mice were assessed in the hot / cold plate test **(a)**, the Hargreaves test **(b)**, and the von Frey test **(c)**. Motor function was assessed using the RotaRod test **(d)**. Data represented as mean \pm SEM, n=16 mice per group. Students t test. Where *, $p < 0.05$ compared to C57Bl/6 mice. All behaviour was carried out blind to genotype.

3.2. Partial sciatic nerve ligation causes comparable behavioural hypersensitivity and histochemical changes in NLRP3^{-/-} mice and C57Bl/6 mice

Baseline mechanical responses in adult NLRP3^{-/-} and C57Bl/6 mice were assessed prior to surgery. PSNL caused a significant and lasting decrease in ipsilateral hindpaw 50% withdrawal threshold to von Frey hairs. Additionally there was a significant decrease in withdrawal latency of the hindpaw in the Hargreaves test. No overall difference was detected in the responses of knockout and wildtype animals ($p=0.07$), although NLRP3^{-/-} mice had a significantly increased withdrawal latency compared to C57Bl/6 mice on day 14, potentially indicating an earlier resolution in thermal hypersensitivity in these animals (Figure 4.3). 14 days after injury a significant increase in the number of microglia in the superficial ipsilateral lumbar dorsal horn was observed, compared to the uninjured contralateral side. This increase was observed in both NLRP3^{-/-} and C57Bl/6 mice, with no difference between the two. Additionally there was no difference observed in the level of activation of microglia between the two genotypes, as assessed by co-localisation with p-p38. As well as an ipsilateral increase in microglial number, there was an increase in astrocyte activation as assessed by a significant increase in the intensity of GFAP immunofluorescence. No difference was observed between NLRP3^{-/-} and C57Bl/6 mice (Figure 4.4).

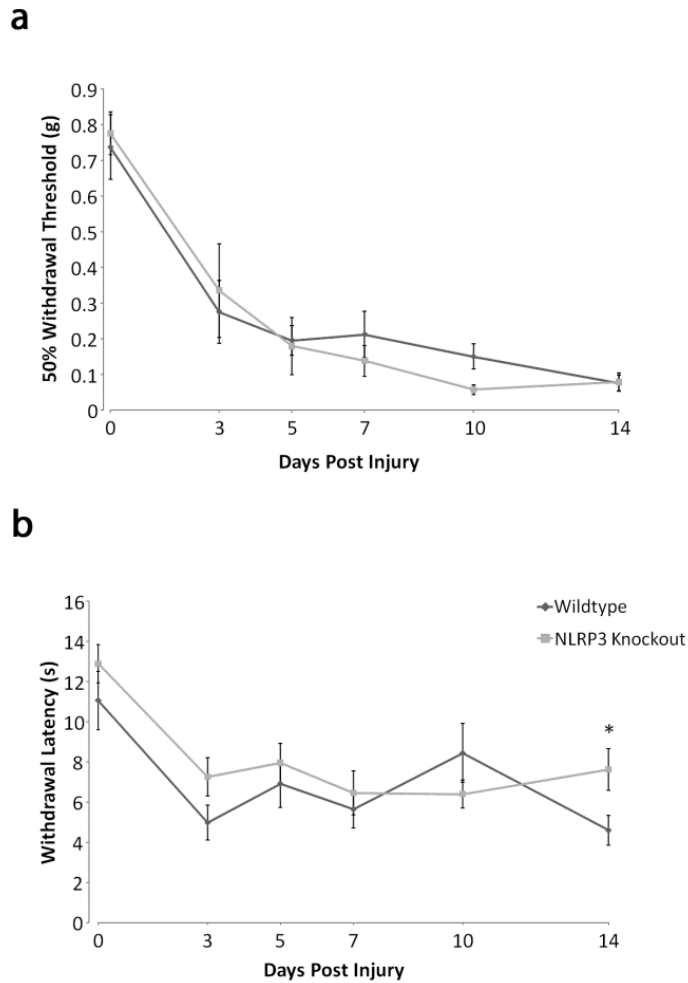


Figure 4.3: Partial sciatic nerve ligation causes comparable behavioural hypersensitivity in NLRP3^{-/-} mice and C57Bl/6 mice

a: PSNL caused mechanical hypersensitivity in the ipsilateral hindpaw in both NLRP3^{-/-} mice and C57Bl/6 mice, with no discernible difference between the two genotypes.

b: PSNL caused a decrease in withdrawal latencies of the ipsilateral hindpaw to a radiant heat stimulus in the Hargreaves plantar test. There was no overall difference in the withdrawal latencies of NLRP3^{-/-} and C57Bl/6 mice ($p=0.07$), although withdrawal latencies of NLRP3^{-/-} mice were significantly increased compared to those of C57Bl/6 mice at day 14. Data expressed as mean \pm SEM, $n=8$ mice per group. Two-way repeated measures ANOVA, with Dunnett's post-test. *, $p<0.05$ compared to C57Bl/6 mice at the same timepoint. All behaviour was carried out blind to genotype.

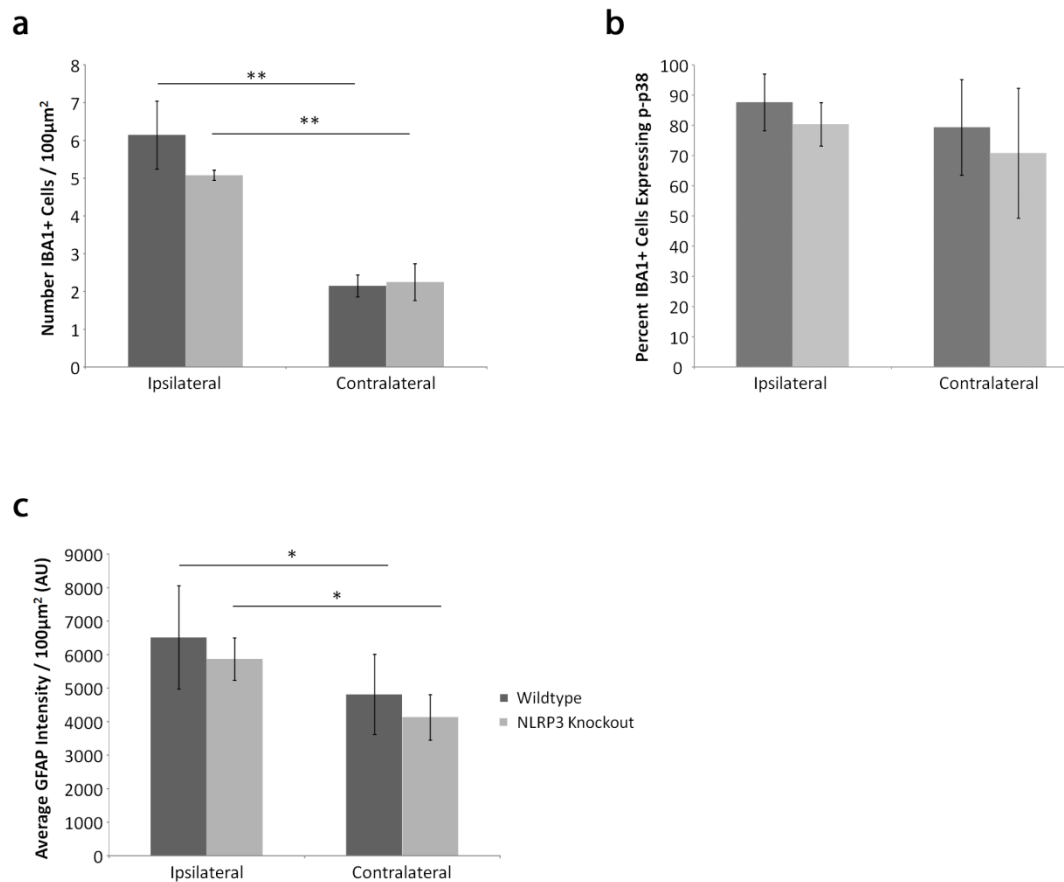


Figure 4.4: No difference in the level of microgliosis or astrocytosis in the superficial dorsal horns of NLRP3^{-/-} mice and C57Bl/6 mice following partial sciatic nerve ligation

a: Immunostaining for microglial marker IBA1 reveals a significant increase in the number of microglia in the superficial ipsilateral lumbar dorsal horn of both NLRP3 knockout and C57Bl/6 mice compared to the contralateral side. No significant difference between the two genotypes was observed. **b:** No difference was observed in the amount of p-p38 co-localisation with IBA1 in superficial dorsal horns of NLRP3^{-/-} mice compared to C57Bl/6 mice. **c:** Immunostaining for astrocyte marker GFAP reveals a significant increase in immunofluorescence in the superficial ipsilateral lumbar dorsal horn compared to the contralateral side in both NLRP3^{-/-} and C57Bl/6 mice. No significant difference between the two genotypes was observed. Data represented as mean \pm SEM, n=3-4 animals per group, with ≥ 3 spinal cord sections analysed per animal. Two-way repeated measures ANOVA with Dunnett's post-test. Where *, ** p<0.05, 0.01 respectively contralateral compared to ipsilateral sides.

3.3. *LPS-induced hindpaw mechanical hypersensitivity is comparable in NLRP3^{-/-} mice and C57Bl/6 mice*

Intrathecal administration of LPS leads to hyperalgesia in the hind-paw (Cahill et al., 2003, Clark et al., 2006, Clark et al., 2010a). Mice were given a priming dose of LPS (2 µg in 5 µl 0.9% sterile saline), followed by a second intrathecal injection of LPS 24 hours later. Mechanical hypersensitivity was assessed 1- and 3 hours later. Following the final behavioural test animals were sacrificed and tissue collected. NLRP3^{-/-} and C57Bl/6 mice showed a significantly reduced mechanical withdrawal threshold from baseline 3 hours after LPS administration. However, there was no difference in NLRP3^{-/-} responses and C57Bl/6 responses at any timepoint (Figure 4.5). Immunohistochemical analysis revealed no difference in the total number of microglia in the superficial lumbar dorsal horn of NLRP3^{-/-} mice and C57Bl/6 mice (average 2.4 ± 0.3 cells/100 µm² and 2.5 ± 0.25 cells/100 µm² in C57Bl/6 and NLRP3^{-/-} animals respectively). The number of microglia expressing p-p38, indicating a state of activation, was not different between the two genotypes ($77.0\% \pm 10.0$ compared to $87.8\% \pm 3.8$ IBA1-positive cells expressing p-p38 in C57Bl/6 and NLRP3^{-/-} mice respectively) (Figure 4.6). No difference was detected in the intensity of GFAP immunoreactivity between treatment groups (data not shown).

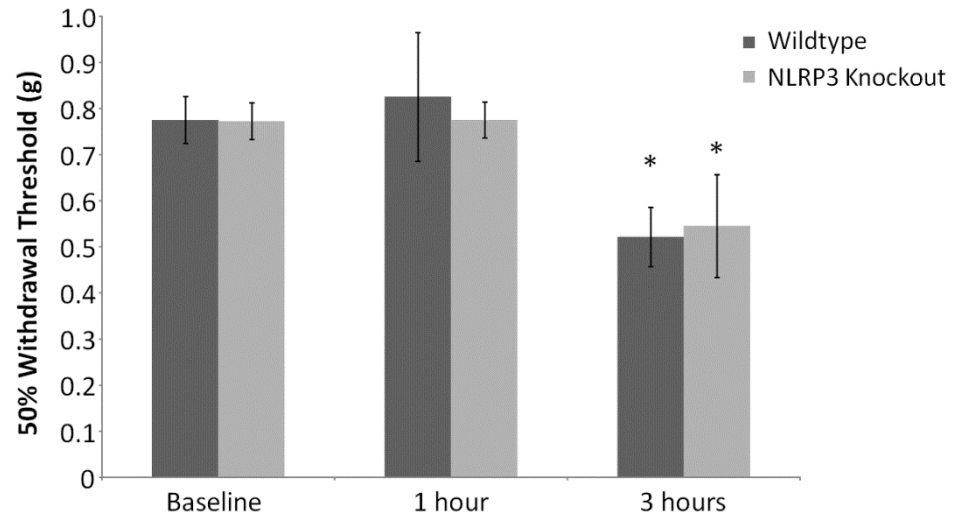


Figure 4.5: No difference in hindpaw mechanical hypersensitivity following intrathecal LPS administration in NLRP3^{-/-} mice compared to C57Bl/6 mice

Intrathecal LPS produces a mechanical hyperalgesia of the hindpaw. Animals received a priming injection of LPS (2 µg in 5 µl 0.9% sterile saline), before receiving a second dose 24 hours. Mechanical hypersensitivity was assessed 1- and 3 hours after the second LPS administration. Data represented as mean ± SEM, n=8 mice per group. Two-way repeated measures ANOVA, followed by Dunnett's post-test, where *, p<0.05 compared to baseline responses. All behaviour was carried out blind to genotype.

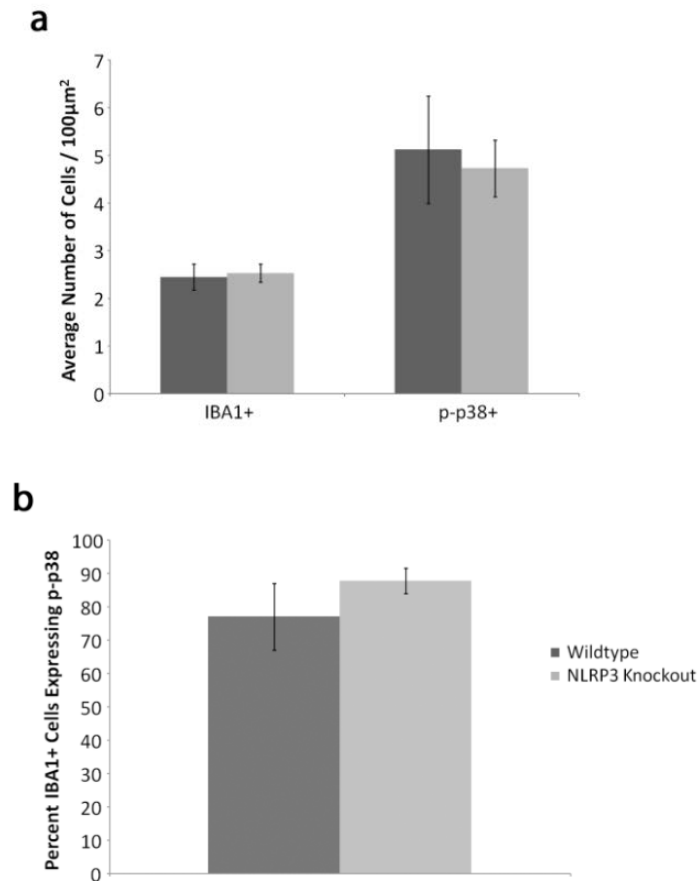


Figure 4.6: No difference in microglial number or activation in NLRP3^{-/-} mice compared to C57Bl/6 mice following intrathecal LPS

a: The number of IBA1⁺ and p-p38⁺ cells in the lumbar dorsal horn did not differ significantly between genotypes. **b:** The proportion of microglia expressing p-p38 was not different between genotypes. Data represented as mean \pm SEM, n=4 mice per group. Two-tailed t test.

4. Discussion

Data presented thus far has suggested that caspase-1 activation contributes to the behavioural hypersensitivity in an animal model of neuropathic pain, independent of the inflammasome. In this chapter NLRP3^{-/-} mice have been used to further clarify this assertion.

Before determining the role of NLRP3 in a model of chronic pain, it was necessary to first determine how, if at all, NLRP3-deficiency affects acute nociceptive signalling. No difference was detected in 50% withdrawal thresholds to punctate mechanical stimuli (von Frey hairs), or in withdrawal latencies to a gradual radiant heating of the plantar surface in the Hargreaves test. However, in the cold- and hotplate test, NLRP3^{-/-} mice showed a significantly increased withdrawal latency at 4°C and 50°C, but not 52°C. This data suggests that NLRP3-deficiency confers a reduced sensitivity to acute noxious thermal stimuli. This is unlikely to be due to a general motor deficit since NLRP3^{-/-} mice had comparable fall latencies in the Rotarod test to C57Bl/6 mice. This result was perhaps surprising given that mice deficient in the inflammasome adaptor protein ASC did not show any differences in withdrawal latencies in the cold- and hotplate tests (see Chapter 3). This indicates that this aspect of NLRP3 function is independent of the adaptor protein ASC. Mutations in the gene encoding the NLRP3 protein have been shown to be the underlying cause of a family of syndromes known as Cryopyrin-Associated Periodic Syndromes (CAPS), characterised by recurrent episodes of symptoms including rash, joint and muscle pain, headaches, fatigue, and fever (Hawkins et al., 2004, Stych and Dobrovolsky, 2008). These symptoms are thought to be due to uncontrolled, excessive, inflammasome activation, leading to inappropriate IL-1 β release (Agostini et al., 2004). There are three described CAPS syndromes; familial cold autoinflammatory syndrome (FACS), Muckle-Wells syndrome, and chronic infantile neurological articular syndrome (CINCA) with increasing severity respectively. Patients with FACS have a distinct trigger for their episodes, namely a cooling of the temperature such as air conditioning, exposure to colder temperatures e.g. during winter months, and ingestion of cold food and drink (Hawkins et al.,

2004, Shinkai et al., 2008). However there is reported overlap of symptoms among the syndromes, and cold can also be a trigger for episodes in Muckle-Wells syndrome and CINCA. Interestingly some patients with NLRP3 mutations also report heat as a trigger factor (Cuisset et al., 2011). Thus it is of interest that mice lacking the NLRP3 protein show altered responses in the cold- and hotplate tests. Investigation into the effect of temperature on the activation of NLRP3 and the mechanism behind cold-induced episodes in patients with NLRP3 mutations has not been reported in the literature however.

In the PSNL model of neuropathic pain no difference was observed in mechanical hypersensitivity and no overall difference in thermal hypersensitivity was observed, although there was a trend for reduced hypersensitivity further indicating a difference in thermal sensitivity in animals lacking the NLRP3 protein. In line with literature reports a significant microgliosis and astrogliosis of the ipsilateral lumbar dorsal horn was observed. No difference in the level of gliosis was observed between genotypes however, as might be expected given the lack of behavioural difference.

The response of NLRP3^{-/-} mice to LPS-induced hypersensitivity was also assessed. In the previous chapter it was shown that, although ASC^{-/-} mice did not show any behavioural differences compared to wildtype littermates in the PSNL model of neuropathic pain, ASC-deficiency conferred some protection against LPS-induced hindpaw mechanical hypersensitivity. ASC is known to associate with NLRP3, following NLRP3 oligomerisation, serving as an adaptor within the inflammasome for pro-caspase-1 recruitment (Martinon et al., 2002, Duncan et al., 2007). Thus it might be expected that NLRP3-deficiency would similarly confer protection from LPS-induced hypersensitivity. However NLRP3^{-/-} mice showed comparable hindpaw hypersensitivity following intrathecal LPS administration to C57Bl/6 mice. This finding is unexpected since ATP stimulation of LPS-primed macrophages and microglia from NLRP3^{-/-} mice leads to a highly blunted release of IL-1 β (Mariathasan et al., 2006, Halle et al., 2008, Hanamsagar et al., 2011). The application of LPS to *ex vivo*

dorsal horn slices results in the release of IL-1 β , active caspase-1, and the inflammasome adaptor protein ASC, suggesting an activation of the inflammasome (Clark et al., 2006). However the sensitivity of NLRP3^{-/-} mice to high doses of LPS leading to endotoxic shock was comparable to wildtype littermates, whereas ASC^{-/-} mice had a significantly higher survival rate compared to wildtype littermates (Sutterwala et al., 2006). NLRP3^{-/-} mice given a lower dose of LPS showed a delay in mortality, and a greater overall survival rate than wildtype littermates suggesting that NLRP3-deficiency confers a degree of protection against LPS-induced mortality to a point. Interestingly, as with ASC^{-/-} mice in the same study, NLRP3^{-/-} mice treated with systemic LPS had significantly reduced serum concentrations of IL-1 β and IL-18. That this was not sufficient to confer protection against endotoxic shock suggests that other inflammatory mediators are released in response to LPS administration, contributing to LPS-induced lethality. Taken together these findings suggest that although NLRP3 is important for LPS-induced IL-1 β release, the effects of LPS application are likely mediated by other, NLRP3-independent, mechanisms. That ASC-deficiency confers greater protection against the effects of LPS, both systemic and intrathecal, suggests a divergence in function of NLRP3 and ASC. There are several emerging reports of ASC acting independently of the inflammasome (Kolly et al., 2009, Ippagunta et al., 2010, Shaw et al., 2010, Ellebedy et al., 2011, Taxman et al., 2011). Potentially, in addition to a role in inflammasome-mediated cytokine release, ASC acts independently of NLRP3 in response to intrathecal LPS administration mediating, in part, LPS-induced hypersensitivity.

It is worth noting that exons 4 and 5 of the NLRP3 gene are not deleted in the NLRP3^{-/-} mouse. Although several bands are visualised when blotting for NLRP3, one band was consistently present in NLRP3^{-/-} spinal cord lysates which was absent from that of C57Bl/6 spinal cord lysates. This may be a truncated form of the protein. If a truncated form of the protein is translated, it is possible that some activity remains.

Another important consideration is that, in this study, the mechanical hypersensitivity observed in wildtype mice in response to LPS was of a smaller

magnitude than that observed in the previous chapter by ASC wildtype mice. This provided a smaller window to observe differences between the two genotypes. Additionally it brings into question the validity of the findings. Confidence in these findings would be increased if the replicate study produced the same findings. Although intrathecal administration of LPS appeared to result in a lesser hypersensitivity in C57Bl/6 mice in this study than that observed in ASC wildtype mice in the study described in the previous chapter, immunohistochemical changes appeared similar. Direct comparison of the number of IBA1+ and p-p38+ cells in the superficial dorsal horn of NLRP3-/- knockout mice was comparable to that observed in C57Bl/6 mice. Additionally there was no difference observed in the proportion of activated microglia, as indicated by co-localisation of IBA1 with p-p38, between the two genotypes.

Data presented in this chapter indicate that NLRP3, and by implication the NLRP3 inflammasome, does not contribute to behavioural hypersensitivity following peripheral nerve injury as an animal model of neuropathic pain. Moreover NLRP3 does not appear to contribute to hypersensitivity in an animal model of central inflammatory pain. However this is not an ideal model of inflammatory pain since it is an acute model, aimed solely at looking at spinal inflammatory processes. Although the aim of this study was to look at the central role of the inflammasome, specifically in glia, it would be of interest to investigate more clinically relevant models of inflammation. Patients with CAPS present with arthropathies, including arthritis. Further investigations of the role of NLRP3 in nociception should perhaps be directed at animal models of arthritic inflammatory pain. Emerging reports suggest that ASC contributes to the pathology of rheumatoid arthritis in an inflammasome-independent manner, although nociceptive outcomes were not assessed in these studies (Kolly et al., 2009, Ippagunta et al., 2010). However there are other reports in which NLRP3 function is implicated in the pathology of rheumatoid arthritis (Rosengren et al., 2005), and also crystal arthritis and Lyme arthritis (Jin et al., 2011, Ray, 2011, Oosting et al., 2012).

Of particular interest, data presented in this chapter indicate that, while NLRP3 appears not to play a role in chronic nociceptive signalling, NLRP3 may play a role in acute nociceptive signalling in response to noxious thermal stimuli. It is known that CAPS patients, in particular FCAS patients, report temperature change as a trigger for inflammatory episodes. Most frequently this trigger is cold, or cooling, stimuli. However, heat has also been reported as a trigger. These findings potentially indicate a temperature-sensitive regulation of NLRP3 signalling. Despite well-documented patient reports of temperature triggers of inflammatory episodes, the underlying molecular mechanism for this phenomenon is unknown, and as yet there are no reports in the literature investigating this aspect of inflammasome signalling.

General Discussion

1. Aims and summary of findings

Immune cell infiltration contributes to the generation and maintenance of behavioural hypersensitivity in animal models of neuropathic and inflammatory pain (Marchand et al., 2005, Calvo et al., 2012). The immune response consists of resident and infiltrating cells which release a variety of inflammatory mediators (Medzhitov, 2008) many of which are capable of eliciting a painful response via direct or indirect actions on the sensory pathway. Among these mediators are the pro-inflammatory cytokines IL-1 β , and also IL-18, both of which are known to be algescic when injected into a variety of tissues (Fukuoka et al., 1994, Cunha et al., 2000). Additionally in animal models of neuropathic and inflammatory pain the expression of IL-1 β message or protein has been shown to increase at various levels of the sensory pathway (Cunha et al., 2000, Milligan et al., 2001, Okamoto et al., 2001, Samad et al., 2001, Lee et al., 2004, Uceyler and Sommer, 2008). Understanding the mechanisms which lead to the maturation and release of these cytokines may provide further opportunity for therapeutic targets, as well as increasing our understanding of those signalling mechanisms contributing to pathologic pain states.

In recent years the mechanisms behind the regulation of IL-1 β , and also IL-18, maturation and release have been elucidated. The first description of the inflammasome was in 2002 when Martinon and colleagues first demonstrated the existence of a multiprotein platform, the NLRP1 inflammasome, for the regulation of IL-1 β release (Martinon et al., 2002). Since this time several other proteins have been proposed to form the central scaffold of inflammasomes including NLRP3, NLRC4, and AIM2. The NLRP3 inflammasome is perhaps the best studied and characterised. Compared to other inflammasome scaffold proteins, the NLRP3 inflammasome has a relatively restricted expression profile, found principally in granulocytes and monocytes and mainly in tissues highly populated in immune cells (Anderson et al., 2004, Kummer et al., 2007, Guarda et al., 2011). It is particularly interesting owing to the large range of activators so far described which, as well as PAMPs, include several DAMPs such as ATP, amyloid- β , and uric acid (Martinon et al., 2009). In recent years a role for the NLRP3 inflammasome has been described in several pathologic states including type II diabetes, gout and pseudo-gout, Alzheimer's disease,

and obesity (Leemans et al., 2011). For these reasons it was considered that the NLRP3 inflammasome may also play a role in chronic pain, known to have a key inflammatory component (Samad et al., 2002, Marchand et al., 2005, Austin and Moalem-Taylor, 2010, Calvo et al., 2012).

There are several lines of evidence which might indicate a role of the inflammasome in both neuropathic and inflammatory pain states. Microglia and macrophages have been strongly implicated as having a role in the development of pain-like behaviours (Liu et al., 2000a, Hu and McLachlan, 2002, Hu et al., 2007, Fu et al., 2009, Xie et al., 2009, Ulmann et al., 2010, Clark et al., 2012). The NLRP3 inflammasome is expressed within macrophages (Petrilli et al., 2007b, Guarda et al., 2011) and microglia (Halle et al., 2008). IL-1 β , as well as activated caspase-1 and ASC, have been shown to be released from LPS-stimulated microglia in *ex-vivo* dorsal horn slices (Clark et al., 2006). P2X7 receptor activation has been shown to cause inflammasome activation in primed cells, and P2X7 receptor signalling in microglia, as well as other cell types, plays a role in pain signalling (Chessell et al., 2005, Honore et al., 2009, Clark et al., 2010a). Increases in intracellular and extracellular calcium have been shown to activate the NLRP3 inflammasome through the calcium-sensing receptor GPRC6A (Lee et al., 2012, Rossol et al., 2012). TLR4 signalling has been shown to play a role in the initiation of neuropathic-pain like behaviours in mice (Tanga et al., 2005). TLR signalling converges to activate transcription factor NF κ B which, in some cellular contexts at least, upregulates pro-IL-1 β and NLRP3 expression (Bauernfeind et al., 2009, Berger et al., 2011). Finally it has been shown that, in several animal models of pain, ROS production, a putative trigger of NLRP3 inflammasome activation, contributes to behavioural hypersensitivity (Wang et al., 2004b, Berger et al., 2011, Salvemini et al., 2011). Thus the aim of the research presented in this thesis was to investigate a possible role for the NLRP3 inflammasome, upstream of IL-1 β maturation and release, in chronic pain.

The main findings of this thesis are:

- The NLRP3 inflammasome is expressed in macrophages and microglia
- Inflammasome component proteins NLRP3, ASC, and active caspase-1 are dysregulated in the L5 SNT model of neuropathic pain in the rat in the injured DRG and ipsilateral lumbar dorsal horn at the mRNA (NLRP3, caspase-1), and protein (ASC, active caspase-1) level
- Caspase-1 inhibition attenuates behavioural hypersensitivity in the L5 SNT model of neuropathic pain, and reduces the activation of inflammatory cells, astrocytes
- This is likely to be inflammasome-independent since ASC^{-/-} and NLRP3^{-/-} mice develop behavioural hypersensitivity in response to partial sciatic nerve ligation injury, in the Seltzer model of neuropathic pain, normally
- In the LPS-induced model of central inflammation caspase-1 inhibition prevent behavioural hypersensitivity and the activation of inflammatory cells, microglia
- This is likely to be ASC-dependent since ASC^{-/-} mice show attenuated behavioural hypersensitivity in response to LPS
- However both are likely to be independent of NLRP3 activation since LPS-induced behavioural hypersensitivity is unaffected in NLRP3^{-/-} mice
- Despite an indication of a role for ASC signalling in a CNS inflammatory model of pain, ASC signalling is dispensable for CFA-induced behavioural hypersensitivity

For many years the behavioural hypersensitivity observed following peripheral nerve injury in animal models of neuropathic pain was considered to be a result of dramatically altered protein expression within the neurons leading to heightened neuronal excitability. However in recent years it has become widely accepted that, even in models of neuropathic pain, there is a key role for the immune system in the initiation and maintenance of pathological pain states. Following peripheral nerve injury there is macrophage infiltration of the DRG,

while centrally microglia migrate and proliferate in the spinal cord at the level of the injury. Evidence suggests these events occur early on, contributing to the initiation of pain states (Tanga et al., 2004, Scholz et al., 2008). It is currently unclear which signalling mechanisms within these cells contribute to behavioural hypersensitivity. In Chapter 1 it was shown that the inflammasome is dysregulated in the injured DRG and ipsilateral lumbar dorsal horn of rats following L5 SNT injury. Further investigation showed that both macrophages and microglia express the inflammasome, and *in vitro* stimulation of these cell types led to NLRP3 upregulation and release of ASC and activated caspase-1 in the extracellular media. However, it was unclear whether the dysregulation of the inflammasome following L5 SNT was due to an upregulation of the inflammasome within macrophages and microglia, or merely a reflection of the increased number of macrophages and microglia following injury..

2. Differential mechanisms of caspase-1 activation in neuropathic and inflammatory pain?

In animal models of both neuropathic and central inflammatory pain caspase-1 inhibition attenuated behavioural hypersensitivity, and reduced the extent of associated histochemical changes with these models. In both cases caspase-1 activation appears to be independent of NLRP3 since NLRP3^{-/-} mice developed behavioural hypersensitivity normally following peripheral nerve injury and intrathecal LPS administration. Interestingly ASC^{-/-} mice developed behavioural hypersensitivity normally following peripheral nerve injury, but not following intrathecal LPS administration demonstrating the activation of different signalling pathways in neuropathic and central inflammatory pain. This is not a simple distinction however since ASC^{-/-} mice developed behavioural sensitivity normally following the peripheral administration of an inflammatory stimulus, CFA.

LPS-induced behavioural hypersensitivity has been shown to be dependent on P2X7 receptor activation, since P2X7^{-/-} mice do not develop behavioural hypersensitivity following intrathecal LPS administration (Clark et al., 2010b). It

was shown that *ex vivo* dorsal horn slices from these animals showed increased extracellular ATP following LPS administration, comparable to wildtype animals, but showed significantly reduced IL-1 β release. This data is consistent with a role for P2X7 receptor activation in the assembly of the NLRP3 inflammasome, thought to be due to the P2X7-mediated K⁺ efflux (Kahlenberg and Dubyak, 2004, Kahlenberg et al., 2005, Qu et al., 2007). Data presented in this thesis show that, *in vitro*, ATP stimulation of LPS-primed microglia leads to the upregulation of NLRP3 protein expression, and an increase in extracellular ASC and active caspase-1. In line with this, following LPS stimulation of *ex vivo* rat dorsal horn slices, it has been shown that, along with IL-1 β , ASC and caspase-1 are secreted (Clark et al., 2006). It is postulated that the release of these proteins allows for the further extracellular maturation of IL-1 β . It is reasonable, therefore, to hypothesise a role of the inflammasome in LPS-induced behavioural hypersensitivity.

In the LPS-induced hypersensitivity model of central inflammatory pain ASC^{-/-} mice showed reduced behavioural hypersensitivity, and a trend towards reduced histochemical changes in the lumbar spinal cord associated with the model. This conferred protection was likely independent of NLRP3, however, since NLRP3^{-/-} mice developed LPS-induced behavioural hypersensitivity normally. ASC was initially characterised as an adaptor protein for the inflammasome (Martinon et al., 2002, Srinivasula et al., 2002). However emerging reports suggest that ASC has several intracellular functions independent of the inflammasome, including a role in pyroptosis, T cell priming in collagen- and antigen-induced arthritis, experimental autoimmune encephalomyelitis, and activation of intracellular MAPK pathways (Kolly et al., 2009, Ippagunta et al., 2010, Shaw et al., 2010, Ellebedy et al., 2011, Taxman et al., 2011). Intrathecal injection of LPS leads to the direct stimulation of microglia via TLR4 receptor activation, a consequence of which is the disinhibition of NF- κ B (Bauernfeind et al., 2009). It has been shown that NF- κ B activation is a necessary “licensing” step for inflammasome activation (Bauernfeind et al., 2009). Interestingly LPS-priming has also been shown to be a necessary step in the formation of pyroptosomes, which are able to form independently of NLRP3 expression (Fernandes-Alnemri et al., 2007). Indeed

it has been postulated that, at least in some situations, NLRP3 may act as a catalyst or enhancer of ASC oligomerisation (Fernandes-Alnemri et al., 2007). Since NLRP3^{-/-} mice develop behavioural hypersensitivity to intrathecal LPS normally, whereas ASC^{-/-} mice are protected, it is possible that ASC oligomerisation is sufficient for caspase-1 activation leading to IL-1 β maturation and release in this model. Alternatively there may be redundancy at the level of the scaffold protein, but not at the level of the adaptor protein. In addition to IL-1 β release, LPS application to spinal microglia *in vitro*, results in the release of nociceptive mediators PGE₂ and NO, in a p38 MAPK-dependent manner (Matsui et al., 2010). Additionally *in vivo* data has shown that intrathecal LPS administration leads to the upregulation of TNF- α and TNF-R in microglia (Shen et al., 2009). *In vitro* it has been shown that microglial release of TNF- α following LPS stimulation occurs downstream of P2X7 receptor activation (Suzuki et al., 2004). Both TNF- α and IL-1 β have been shown to be capable of priming the inflammasome in a manner similar to LPS, possibly representing a positive feedback loop enhancing the release of IL-1 β (Franchi et al., 2009).

Since ASC^{-/-} mice showed reduced sensitivity in the LPS-induced model of inflammatory pain, it was postulated that ASC may play a role in inflammatory pain. However ASC^{-/-} mice did not show any difference in behavioural hypersensitivity compared to wildtype mice following intraplantar administration of CFA. Owing to time and resource restrictions, the role of other inflammasome components in this model of inflammatory pain was not tested. However it has been shown that IL-1 $\alpha\beta$ ^{-/-} mice show a reduction in behavioural hypersensitivity following CFA administration, possibly indicating a role of IL-1 β maturation and release in this model (Honore et al., 2006, Honore et al., 2009). However data presented in this thesis indicate an inflammasome-independent role.

Finally an ASC- and NLRP3-independent mechanism of caspase-1 activation is apparent following peripheral nerve injury as an animal model of neuropathic pain. That ASC does not contribute to behavioural hypersensitivity in all animal models of pain tested is perhaps not surprising. Several reports have been

published looking at differences in animal models of neuropathic and inflammatory pain. While there are some changes in common, such as an increased production of cytokines, the other changes are not (Honore et al., 2000, Yajima et al., 2003, Zhao et al., 2006, Abrahamsen et al., 2008). For example mice lacking sodium channel Nav1.8 expression in sensory neurons show a reduction in behavioural hypersensitivity in response to inflammatory stimuli, but not following peripheral nerve injury (Abrahamsen et al., 2008). Similarly selective knockout of BDNF expression from sensory neurons results in a reduction in behavioural hypersensitivity to inflammatory stimuli, but not following peripheral nerve injury (Zhao et al., 2006). These findings reflect the different pathophysiological mechanisms underlying inflammatory and neuropathic pain.

3. Redundancy and the innate immune response

The innate immune system can be considered as an organism's first line of defence against invading microorganisms and pathogens. It is an ancient, evolutionarily-conserved series of host mechanisms which allow detection and defence in a non-specific manner (Medzhitov and Janeway, 2000, Janeway and Medzhitov, 2002). A major aspect of the innate immune system is the recognition of pathogen-associated molecular patterns, PAMPs. These are conserved molecular patterns not expressed by the host, but commonly expressed by invading microorganisms such as peptidoglycan or lipopolysaccharide (LPS). The host is able to recognise multiple PAMPs, and via multiple mechanisms, generating redundancy in the system which confers a greater level of host protection.

Data presented in this thesis demonstrates a role for caspase-1 in animal models of neuropathic and central inflammatory pain. However this appears to be inflammasome-independent in the case of neuropathic pain, and NLRP3-independent in the case of central inflammatory pain. When using transgenic mice, the possibility of some level of compensation should always be considered. In the case of the inflammasome and innate immunity, some level of redundancy in the system is expected given the critical role of innate

immunity. Indeed it has been shown that caspase-1, IL-1 β , and IL-18 knockout mice are more susceptible to infection by *Salmonella typhimurium* with a reduced survival time, whereas mice deficient in NLRP3, NLRC4, and ASC are not (Lara-Tejero et al., 2006, Raupach et al., 2006). Similar results were obtained by Broz *et al.* who showed redundancy in NLR activation and, to a lesser extent, ASC in response to *S. typhimurium* (Broz et al., 2010). Additionally a level of redundancy in inflammasome activation has been demonstrated following infection with *Listeria monocytogenes* (Kim et al., 2010b, Wu et al., 2010). Thus, as far as pathogen recognition is concerned, there is a level of redundancy among the inflammasomes. There is scope for such redundancy to occur not only at the level of the scaffold protein, but also, to some extent, at the level of the adaptor protein.

The NLR family is characterised by a tripartite structure, consisting of an N-terminal protein-protein interaction domain (a CARD domain, a PYRIN domain, acidic transactivating domain, or a baculovirus inhibitor repeat (BIR)), a central nucleotide-binding oligomerisation domain (NOD), and a C-terminal LRR domain. In humans there are 22 members of the NLR family, and in mice there are at least 33 NLR genes (Chen et al., 2009). Thus it is perhaps not surprising to find redundancy in this system. NLRP1-14 all contain a PYRIN domain, while several proteins of this family contain a CARD domain, including CIITA, NOD1, NOD2, NLRC4, NLRC5, and NLRP1, all of which could potentially interact with caspase-1 (Chen et al., 2009, Schroder and Tschopp, 2010). However this argument does not take into account the expression patterns of these proteins, or particular physiological conditions normally required for their activation. Expression of a PYRIN or CARD domain is not necessarily sufficient for protein-protein interactions. Bruey and colleagues have shown that NLRP2 is able to bind to the PYRIN domain of NLRP1 but not the full-length NLRP1 suggesting that PYRIN domains may be in some way masked, limiting interactions (Bruey et al., 2004).

NLRP2, expression of which is inducible following LPS stimulation of macrophages, has been shown to associate with the adaptor protein ASC and form a functional IL-1 β processing inflammasome (Agostini et al., 2004, Bruey

et al., 2004). NLRP12 has also been shown to associate with the adaptor protein ASC and enhance the activation of pro-caspase-1 and subsequent maturation and release of IL-1 β (Wang et al., 2002). NLRP1 and NLRC4, also able to form functional IL-1 β processing inflammasomes, possess a CARD domain and are therefore able to interact directly with caspase-1. Whereas NLRP2 and NLRP12, like NLRP3, are able to enhance caspase-1-mediated processing of IL-1 β in response to LPS, NLRP1 and NLRC4 do not appear to assemble following LPS stimulation (Mariathasan et al., 2004, Faustin et al., 2007). In fact NLRP1 and NLRC4 appear to have a more restricted range of activators, limited to PAMPs, compared to NLRP3 (Martinon et al., 2007, Martinon et al., 2009). Little is known about the physiological agonists of NLRP2 and NLRP12 inflammasomes, however. Evidence suggests a role for TLR4 activation in microglia, the receptor at which LPS exerts its effects, in neuropathic pain (Tanga et al., 2005, Bettoni et al., 2008, Cao et al., 2009). Thus inflammasomes which assemble in response to LPS stimulation may perhaps be considered as candidates for alternative mechanisms of caspase-1 activation.

Activation of Nod1, an intracellular pattern recognition receptor (PRR), leads to its oligomerisation and subsequent recruitment of its CARD-containing adaptor protein RIP2. RIP2 then oligomerises and interacts with the IKK complex, ultimately leading to the release and nuclear translocation of NF- κ B (Inohara et al., 2000). Nod1 has been shown to enhance LPS-induced IL-1 β release via its interaction with pro-caspase-1, apparently independent of TLR4 activation. Indeed Nod1 is activated by the presence of breakdown products of peptidoglycans, including LPS (Yoo et al., 2002, Khare et al., 2010, Kavathas et al., 2012). Consequently Nod1 is unlikely to play a role in animal models of neuropathic pain. The adaptor protein RIP2 has also been shown to directly interact with pro-caspase-1, an association that is enhanced following LPS stimulation (Thome et al., 1998, Humke et al., 2000, Zhang et al., 2003). In addition to its ability to associate with Nod1, RIP2 has also been suggested to act downstream of TNF receptor activation via an association with the adaptor proteins TRAF1 and TRAF2 (Thome et al., 1998). Increased TNF- α expression in the periphery and centrally has been demonstrated in animal models of

inflammatory and neuropathic pain (Kiguchi et al., 2008, Shen et al., 2009, Leung and Cahill, 2010, Zhang et al., 2011). Thus RIP2 represents a possible alternative pathway for the activation of caspase-1.

In an independent study, carried out by Dr. Ana Antunes-Martins, next generation RNA sequencing techniques were employed to examine changes in gene expression in the ipsilateral lumbar dorsal horn and L5 DRG of rats following L5 SNT injury. Data from this study demonstrated that the genes encoding CARD-containing proteins NLRP1 and NLRC4 were significantly upregulated in the ipsilateral lumbar dorsal horn of rats 7 days after SNT injury. The genes encoding NLRP1, NLRC4, and Nod1 were similarly upregulated in the L5 DRG 7 days after injury, lending support to the idea of redundancy in the activation of caspase-1. Interestingly the genes encoding CARD-containing proteins NLRP12 and RIP2 were shown not to be dysregulated following L5 SNT injury, and so it is perhaps less likely that these proteins were compensating for the loss of ASC or NLRP3 following PNL injury,

IL-1 β has far-reaching physiological effects and subsequently its maturation and release is under tight control, as discussed in the Introduction. Perhaps unsurprisingly, then, there are alternative mechanisms of IL- β maturation and release. Leptin is a small peptide hormone, mainly secreted by adipose tissue. Leptin has been implicated in the inflammatory process through its actions at the Ob receptor (ObR) expressed on cells of the innate and adaptive immune response including T and B cells, macrophages, and haemopoietic cells (Matarese et al., 2005, Bernotiene et al., 2006). Leptin-stimulated macrophages and monocytes have been shown to up-regulate phagocytic function and pro-inflammatory cytokine secretion (Gainsford et al., 1996). More recently leptin-induced mature IL-1 β secretion from microglia was shown to be via a caspase-1-independent mechanism (Pinteaux et al., 2007). Interestingly a role for leptin in animal models of neuropathic pain has been suggested. Following partial sciatic nerve ligation leptin has been shown to increase in adipocytes in the proximity of the injured nerve. Mice deficient in the ObR failed to develop peripheral nerve injury-induced behavioural hypersensitivity, a phenotype which was reversed upon administration of either leptin or leptin-

treated macrophages to the injured nerve (Maeda et al., 2009). Following peripheral nerve injury expression of the ObR has been shown to be significantly upregulated in the ipsilateral lumbar dorsal horn. Spinal administration of a leptin antagonist has been shown to both prevent and reverse peripheral nerve injury-induced behavioural hypersensitivity, while intrathecal administration of leptin to naïve rats induced behavioural hypersensitivity (Lim et al., 2009).

Matrix metalloproteases (MMPs) are extracellular proteases which participate in tissue remodelling via the breakdown of extracellular matrix proteins. MMPs play a key role in the inflammatory response. Following peripheral nerve injury TNF- α and IL-1 β secreted by Schwann cells and resident mast cells induces the production of MMP-9 in Schwann cells, which degrades myelin basic protein (MBP) and promotes macrophage recruitment (Chattopadhyay et al., 2007, Kobayashi et al., 2008a). MMPs are also able to cleave chemokines and cytokines, including IL-1 β (Schonbeck et al., 1998, Parks et al., 2004). Following peripheral nerve injury, IL-1 β cleavage has been shown to occur through the actions of MMP-9 and MMP-2 in the early and later phases of the model, respectively (Kawasaki et al., 2008a). In addition to leptin and MMPs, chymase and elastase, both mast cell and neutrophil-derived proteases, have been implicated in IL-1 β maturation and release in an animal model of arthritis (Guma et al., 2009).

As well as the need for redundancy in sensor proteins and cytokine maturation mechanisms, there is also redundancy in the mediators of the inflammatory response, many of which have effects in common. Although much evidence points to an algescic role of IL-1 β as a result of an inflammatory response, in neuropathic and inflammatory pain states, it is only one of several pro-inflammatory cytokines shown to play a key role in the inflammatory response. The prototypical pro-inflammatory cytokines TNF- α and IL-6 are also widely reported to contribute to pain-like behaviours in animal models of inflammatory and neuropathic pain (Cui et al., 2000, Ozaktay et al., 2006, Schoeniger-Skinner et al., 2007, Kawasaki et al., 2008b, Kiguchi et al., 2008, Shen et al., 2009, Lee et al., 2010a, Leung and Cahill, 2010, Zhang et al., 2011). TNF- α ,

IL- β , and IL-6 are all able to sensitise sensory neurons either by increasing excitatory synaptic transmission or by decreasing inhibitory synaptic transmission of sensory neurons (Ozaktay et al., 2006, Kawasaki et al., 2008b). Interestingly the pro-nociceptive actions of these cytokines appears to be synergistic. Combinatorial treatment aimed at reducing both IL-1R and TNF-R signalling was found to be more efficacious at reducing peripheral nerve injury-induced hypersensitivity than either treatment administered alone (Schafers et al., 2001, Sweitzer et al., 2001). Additionally IL-1R and TNF-R signalling converges at the level of TAK1, an activating kinase for the IKK complex, ultimately leading to the ubiquitination and degradation of I κ B allowing NF- κ B to translocate to the nucleus (Holtmann et al., 2001). Consequently reduction of IL-1 β maturation and release alone may not be sufficient for reduction of peripheral nerve injury-induced hypersensitivity.

Blockade of IL-1 β signalling in neuropathic pain has been shown to be only mildly efficacious, or ineffective. In line with the concept of redundancy in the inflammatory response, combination treatment with a therapy which inhibits TNF- α actions results in synergistic reductions in peripheral nerve injury-induced hypersensitivity (Schafers et al., 2001, Sweitzer et al., 2001). It is postulated that joint reduction of these cytokines reduces the subsequent upregulation of IL-6, downstream of both signalling pathways. Potentially attempts at reducing IL-1 β signalling through the knockout of inflammasome component proteins are not sufficient to alter behavioural hypersensitivity owing to the compensatory actions of TNF- α . The differing relative contributions of these cytokines, and indeed other mechanisms, may be reflected in the differing responses of ASC-/- mice in the different animal models of pain. Caspase-1 inhibition led to a partial reversal of mechanical hypersensitivity following L5 SNT injury. However ASC-/- mice developed behavioural hypersensitivity normally following Seltzer injury. This discrepancy may lie in a developmental compensation. Indeed treatment with CRID3, an inhibitor of ASC oligomerisation, led to a small but significant reversal of SNT-induced mechanical hypersensitivity in rats.

4. Future directions

There currently exist few pharmacological options for the inhibition of the inflammasome. This is due, in part, to the nature of assembly and activation via homotypic protein-protein interactions. Inhibition of these interactions is unlikely to be specific to the inflammasome and could disrupt several intracellular signalling pathways. The sulfonylurea anti-diabetic drug glyburide has been shown to inhibit the inflammasome, via an action that is independent of its actions on ATP-sensitive potassium channels (Lamkanfi et al., 2009). The anti-inflammatory compounds parthenolide and Bay11-7082 also inhibit inflammasome activation independently of their effects on NF- κ B inhibition (Juliana et al., 2010). Although these actions appear to be inflammasome-specific, these compounds have other actions which are unrelated to the inflammasome, and so are not a suitable option for treatment. Indeed the treatment for CAPS patients is not targeted at the inflammasome, but rather the downstream effect of excessive activation; IL-1 β release. Anakinra, rilonacept, and canakinumab, recombinant IL-1Ra, soluble IL-1R, and an antibody against IL-1 β respectively, are all highly effective therapies in CAPS (Dinarello, 2011). Interestingly anakinra has proved to be an effective drug in rheumatoid arthritis both in terms of disease-state and pain (Mertens and Singh, 2009). Although administration of IL-1Ra in rodents following peripheral nerve injury has been shown to reduce behavioural hypersensitivity, administration in combination with a treatment for blocking the actions of TNF- α has been shown to be more efficacious (Schafers et al., 2001, Sweitzer et al., 2001, Winkelstein et al., 2001). There are no reports on the effect of either IL-1Ra or a combinatorial therapy in humans for neuropathic pain however. Therapy directed towards reducing the level of pro-inflammatory cytokines may be of some benefit. However therapy directed at inhibiting pathways upstream of cytokine production and release may not be. Data presented in this thesis point towards a complex system of redundancy within the innate immune system, and so it is preferable to target the final common output. Further the innate immune response is a critical first-line defence against invading microorganisms, with the inflammasome seemingly playing a key role. As such, pharmacological inhibition of such a system may be more harmful than beneficial to the individual.

Appendix

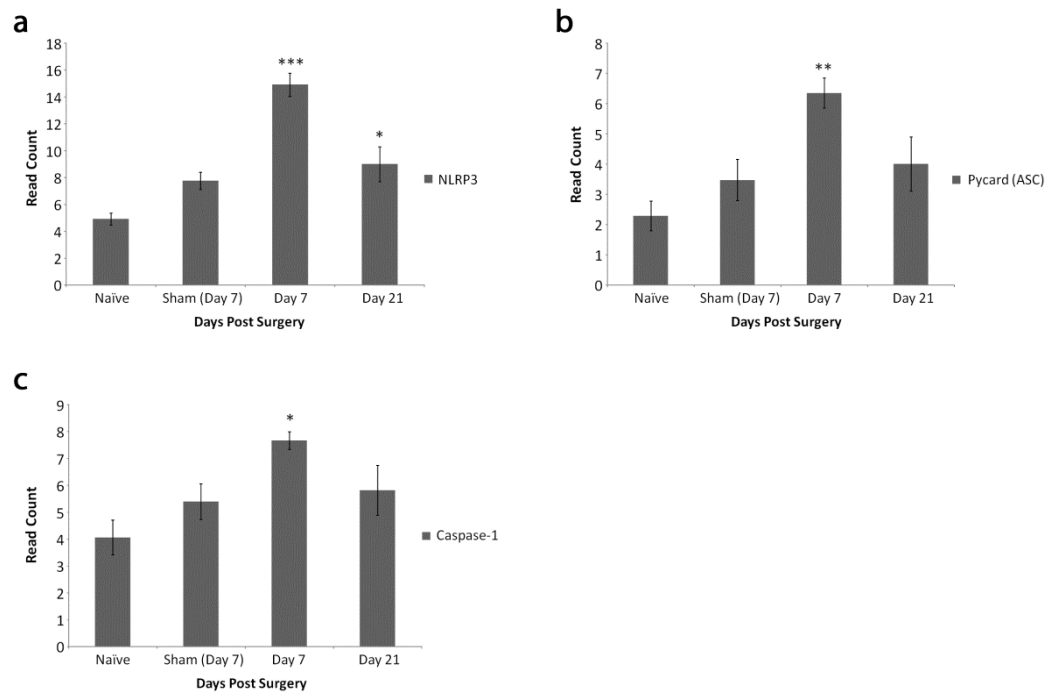


Figure a.1: Inflammasome component genes NLRP3, Pycard (ASC), and caspase-1 are dysregulated in the ipsilateral lumbar dorsal horn after L5 SNT

RNA-sequencing of ipsilateral lumbar dorsal horns of rats following L5 SNT injury reveals a significant increase in gene expression of inflammasome components NLRP3 **(a)** Pycard (ASC) **(b)**, and caspase-1 **(c)**. One-way ANOVA with Dunnett's post-test, where *,** $p < 0.05, 0.01$ respectively, compared to naïve animals. $n=3-4$ per group.

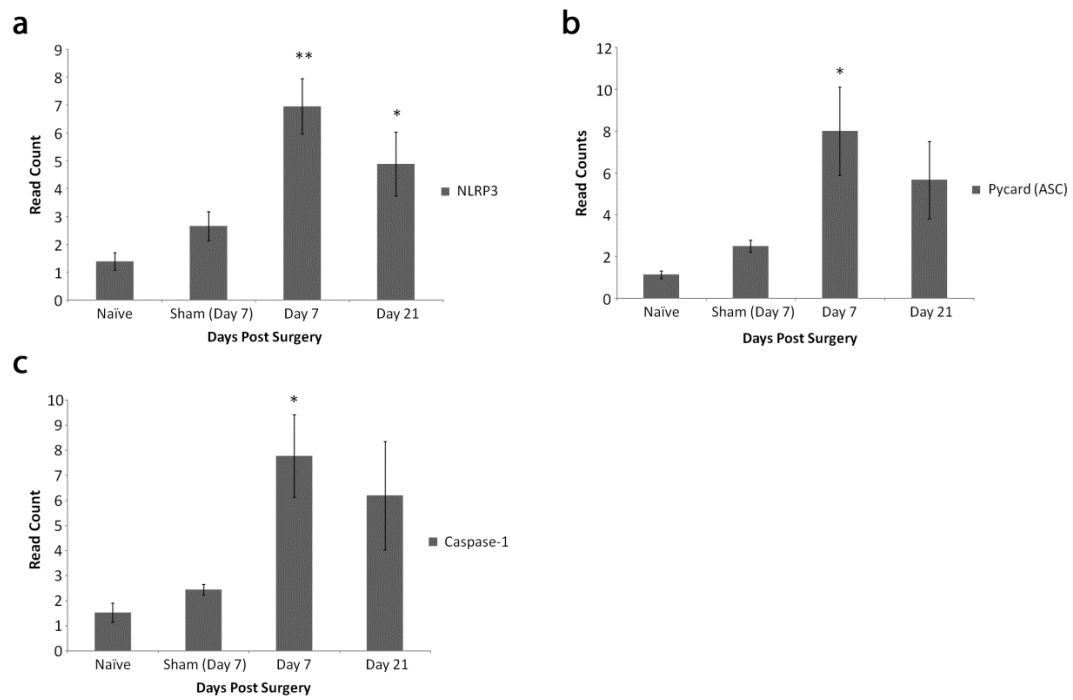


Figure a.2: Inflammasome component genes NLRP3, Pycard (ASC), and caspase-1 are dysregulated in the L5 DRG after L5 SNT

RNA-sequencing of injured L5 DRG of rats following L5 SNT injury reveals a significant increase in gene expression of inflammasome components NLRP3 **(a)** Pycard (ASC) **(b)**, and caspase-1 **(c)**. One-way ANOVA with Dunnett's post-test, where *,** $p < 0.05, 0.01$ respectively, compared to naïve animals. $n=3-4$ per group.

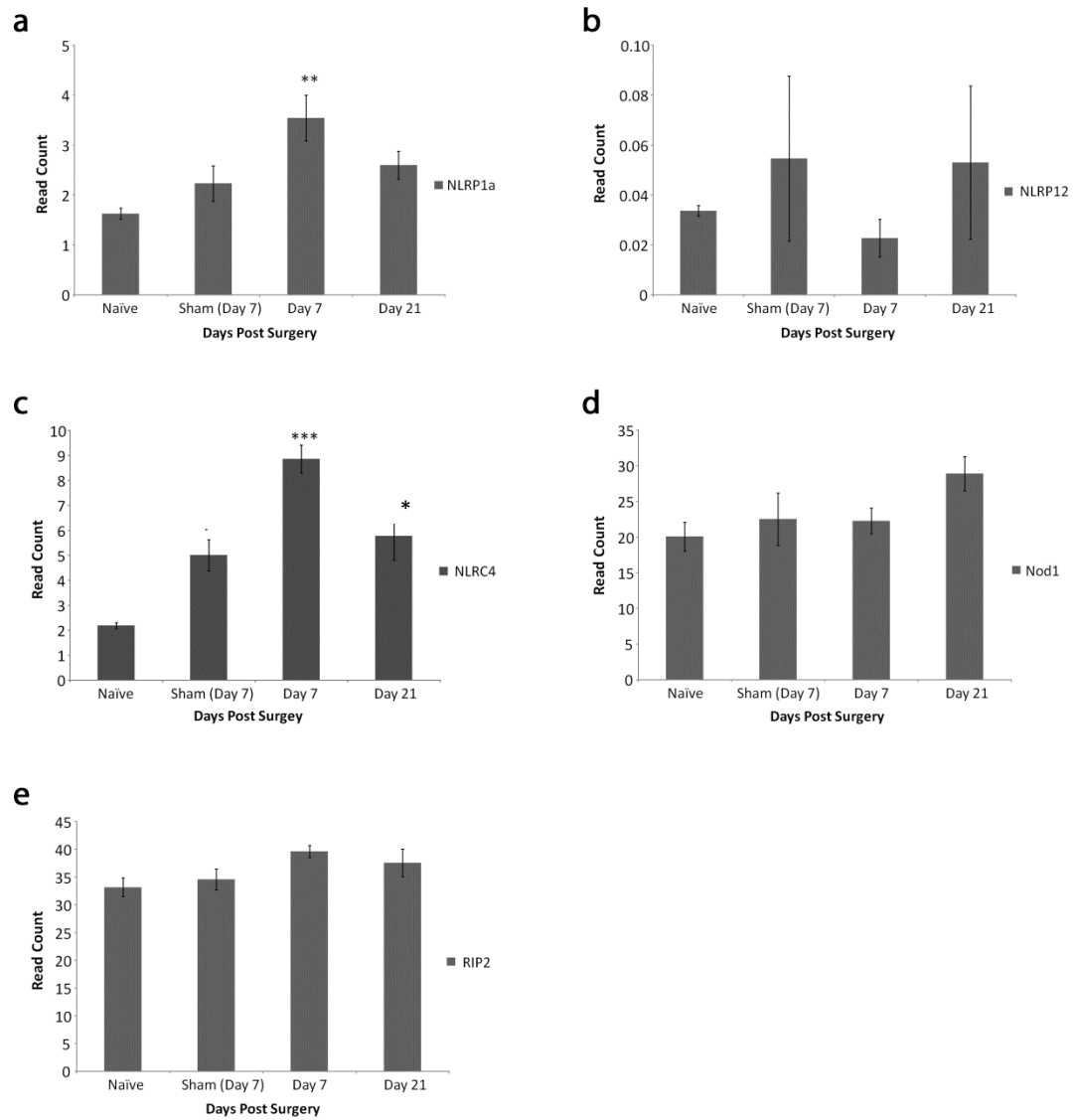


Figure a.3: CARD domain-containing proteins NLRP1, NLRP12, NLRC4, Nod1, and RIP2 genes are dysregulated in the ipsilateral lumbar dorsal horn after L5 SNT

RNA-sequencing of ipsilateral lumbar dorsal horns of rats following L5 SNT injury reveals a significant increase in gene expression of CARD domain-containing proteins NLRP1a (a), NLRP12 (b), NLRC4 (c), Nod1 (d), and RIP2 (e). One-way ANOVA with Dunnett's post-test, where *, **, *** $p < 0.05, 0.01, 0.001$ respectively, compared to naïve animals. $n=3-4$ per group.

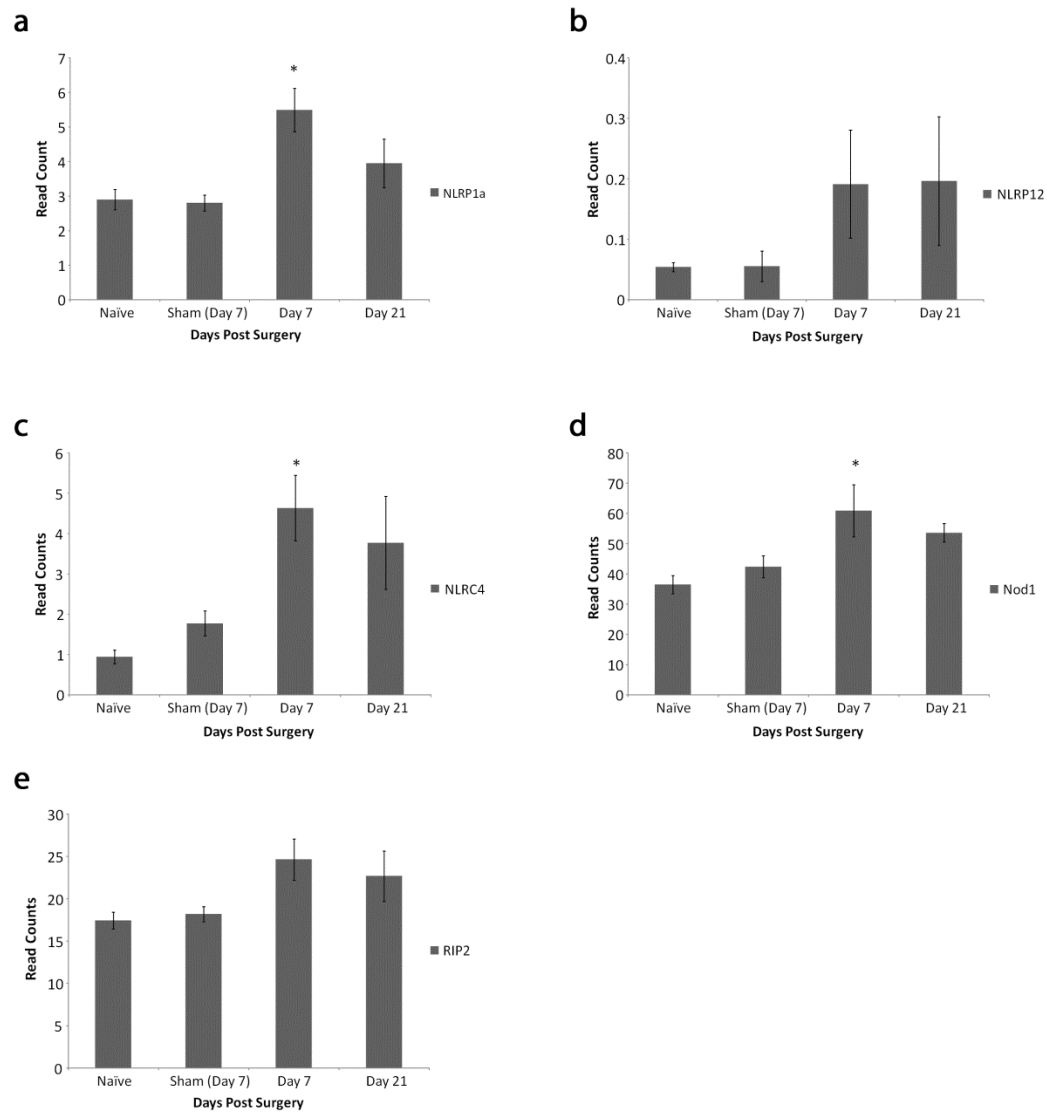


Figure a.4: CARD domain-containing proteins NLRP1, NLRP12, NLRC4, Nod1, and RIP2 genes are dysregulated in L5 DRG after L5 SNT

RNA-sequencing of injured L5 DRG of rats following L5 SNT injury reveals a significant increase in gene expression of CARD domain-containing proteins NLRP1a (a), NLRP12 (b), NLRC4 (c), Nod1 (d), and RIP2 (e). One-way ANOVA with Dunnett's post-test, where * $p < 0.05$, compared to naïve animals. $n = 3-4$ per group.

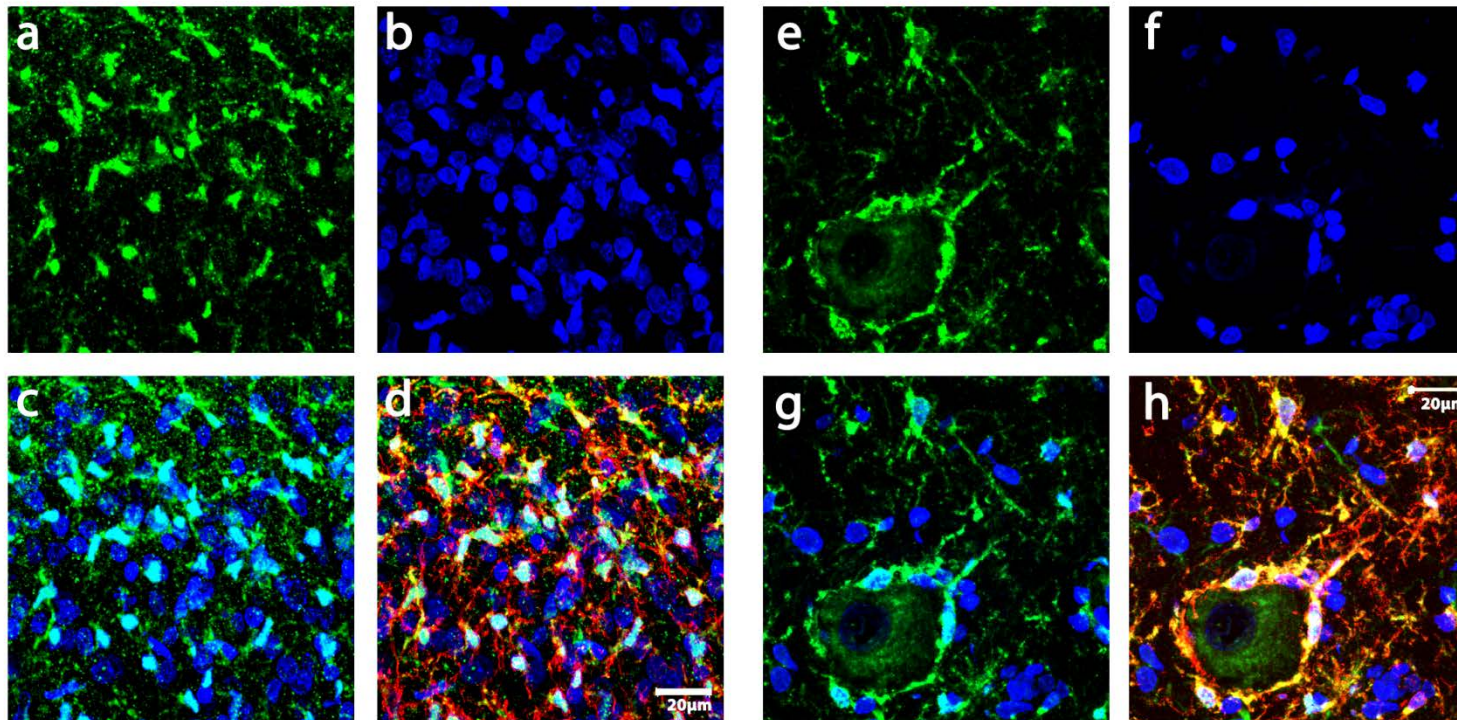


Figure a.5: Caspase-1 immunohistochemistry within the rat spinal cord

a – d: Representative image of caspase-1 immunostaining within the ipsilateral dorsal horn of a rat 7 days after L5 SNT. **e – h:** Representative image of caspase-1 immunostaining within the ipsilateral ventral horn of a rat 7 days after L5 SNT. Co-localisation of caspase-1 with nuclear marker DAPI can be seen in panels **c** and **g**. Caspase-1 is represented in the green channel, DAPI is represented in the blue channel, and IBA1 is represented in the red channel. Scale 20 μm .

References

- Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, Forrest MJ (2003) Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc Natl Acad Sci U S A* 100:7947-7952.
- Abrahamsen B, Zhao J, Asante CO, Cendan CM, Marsh S, Martinez-Barbera JP, Nassar MA, Dickenson AH, Wood JN (2008) The cell and molecular basis of mechanical, cold, and inflammatory pain. *Science* 321:702-705.
- Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J (2004) NALP3 forms an IL-1 β -processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20:319-325.
- Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU (2002) PGE(2) selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. *Nature neuroscience* 5:34-40.
- Aizawa Y, Akita K, Taniai M, Torigoe K, Mori T, Nishida Y, Ushio S, Nukada Y, Tanimoto T, Ikegami H, Ikeda M, Kurimoto M (1999) Cloning and expression of interleukin-18 binding protein. *FEBS letters* 445:338-342.
- Akira S, Takeda K (2004) Toll-like receptor signalling. *Nature reviews Immunology* 4:499-511.
- Aliprantis AO, Yang RB, Weiss DS, Godowski P, Zychlinsky A (2000) The apoptotic signaling pathway activated by Toll-like receptor-2. *The EMBO journal* 19:3325-3336.
- Allen AL, Cortright DN, McCarson KE (2003) Formalin- or adjuvant-induced peripheral inflammation increases neurokinin-1 receptor gene expression in the mouse. *Brain Res* 961:147-152.
- Allodi I, Uchina E, Navarro X (2012) Specificity of peripheral nerve regeneration: interactions at the axon level. *Prog Neurobiol* 98:16-37.
- Alzabin S, Williams RO (2011) Effector T cells in rheumatoid arthritis: lessons from animal models. *FEBS letters* 585:3649-3659.
- Amaya F, Shimosato G, Nagano M, Ueda M, Hashimoto S, Tanaka Y, Suzuki H, Tanaka M (2004) NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. *Eur J Neurosci* 20:2303-2310.
- Anderson JP, Mueller JL, Rosengren S, Boyle DL, Schaner P, Cannon SB, Goodyear CS, Hoffman HM (2004) Structural, expression, and evolutionary analysis of mouse CIAS1. *Gene* 338:25-34.

- Andrei C, Margiocco P, Poggi A, Lotti LV, Torrisi MR, Rubartelli A (2004) Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. *Proc Natl Acad Sci U S A* 101:9745-9750.
- Arend WP, Palmer G, Gabay C (2008) IL-1, IL-18, and IL-33 families of cytokines. *Immunological reviews* 223:20-38.
- Austin PJ, Moalem-Taylor G (2010) The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. *Journal of neuroimmunology* 229:26-50.
- Barclay J, Clark AK, Ganju P, Gentry C, Patel S, Wotherspoon G, Buxton F, Song C, Ullah J, Winter J, Fox A, Bevan S, Malcangio M (2007) Role of the cysteine protease cathepsin S in neuropathic hyperalgesia. *Pain* 130:225-234.
- Baron R (2006) Mechanisms of disease: neuropathic pain--a clinical perspective. *Nature clinical practice Neurology* 2:95-106.
- Baron R, Tolle TR, Gockel U, Brosz M, Freynhagen R (2009) A cross-sectional cohort survey in 2100 patients with painful diabetic neuropathy and postherpetic neuralgia: Differences in demographic data and sensory symptoms. *Pain* 146:34-40.
- Barrot M (2012) Tests and models of nociception and pain in rodents. *Neuroscience* 211:39-50.
- Basbaum AI, Bautista DM, Scherrer G, Julius D (2009) Cellular and molecular mechanisms of pain. *Cell* 139:267-284.
- Bauer CS, Nieto-Rostro M, Rahman W, Tran-Van-Minh A, Ferron L, Douglas L, Kadurin I, Sri Ranjan Y, Fernandez-Alacid L, Millar NS, Dickenson AH, Lujan R, Dolphin AC (2009) The increased trafficking of the calcium channel subunit alpha2delta-1 to presynaptic terminals in neuropathic pain is inhibited by the alpha2delta ligand pregabalin. *J Neurosci* 29:4076-4088.
- Bauernfeind F, Bartok E, Rieger A, Franchi L, Nunez G, Hornung V (2011) Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *Journal of immunology* 187:613-617.
- Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *Journal of immunology* 183:787-791.
- Bee LA, Dickenson AH (2007) Rostral ventromedial medulla control of spinal sensory processing in normal and pathophysiological states. *Neuroscience* 147:786-793.

- Beggs S, Liu XJ, Kwan C, Salter MW (2010) Peripheral nerve injury and TRPV1-expressing primary afferent C-fibers cause opening of the blood-brain barrier. *Mol Pain* 6:74.
- Beggs S, Trang T, Salter MW (2012) P2X4R+ microglia drive neuropathic pain. *Nature neuroscience* 15:1068-1073.
- Beiche F, Scheuerer S, Brune K, Geisslinger G, Goppelt-Strube M (1996) Up-regulation of cyclooxygenase-2 mRNA in the rat spinal cord following peripheral inflammation. *FEBS letters* 390:165-169.
- Bennett DL, French J, Priestley JV, McMahon SB (1996) NGF but not NT-3 or BDNF prevents the A fiber sprouting into lamina II of the spinal cord that occurs following axotomy. *Mol Cell Neurosci* 8:211-220.
- Bennett DL, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J Neurosci* 18:3059-3072.
- Bennett GJ, Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33:87-107.
- Berger JV, Deumens R, Goursaud S, Schafer S, Lavand'homme P, Joosten EA, Hermans E (2011) Enhanced neuroinflammation and pain hypersensitivity after peripheral nerve injury in rats expressing mutated superoxide dismutase 1. *Journal of neuroinflammation* 8:33.
- Bernotiene E, Palmer G, Gabay C (2006) The role of leptin in innate and adaptive immune responses. *Arthritis research & therapy* 8:217.
- Bettoni I, Comelli F, Rossini C, Granucci F, Giagnoni G, Peri F, Costa B (2008) Glial TLR4 receptor as new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. *Glia* 56:1312-1319.
- Biber K, Sauter A, Brouwer N, Copray SC, Boddeke HW (2001) Ischemia-induced neuronal expression of the microglia attracting chemokine Secondary Lymphoid-tissue Chemokine (SLC). *Glia* 34:121-133.
- Biber K, Tsuda M, Tozaki-Saitoh H, Tsukamoto K, Toyomitsu E, Masuda T, Boddeke H, Inoue K (2011) Neuronal CCL21 up-regulates microglia P2X4 expression and initiates neuropathic pain development. *The EMBO journal* 30:1864-1873.
- Black JA, Liu S, Tanaka M, Cummins TR, Waxman SG (2004) Changes in the expression of tetrodotoxin-sensitive sodium channels within dorsal root ganglia neurons in inflammatory pain. *Pain* 108:237-247.
- Blackbeard J, Wallace VC, O'Dea KP, Hasnie F, Segerdahl A, Pheby T, Field MJ, Takata M, Rice AS (2012) The correlation between pain-related behaviour and spinal microgliosis in

- four distinct models of peripheral neuropathy. *European journal of pain* 16:1357-1367.
- Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS (2003) A unified model for apical caspase activation. *Molecular cell* 11:529-541.
- Boatright KM, Salvesen GS (2003) Mechanisms of caspase activation. *Current opinion in cell biology* 15:725-731.
- Boucher TJ, McMahon SB (2001) Neurotrophic factors and neuropathic pain. *Curr Opin Pharmacol* 1:66-72.
- Boucher TJ, Okuse K, Bennett DL, Munson JB, Wood JN, McMahon SB (2000) Potent analgesic effects of GDNF in neuropathic pain states. *Science* 290:124-127.
- Boue J, Blanpied C, Brousset P, Vergnolle N, Dietrich G (2011) Endogenous opioid-mediated analgesia is dependent on adaptive T cell response in mice. *Journal of immunology* 186:5078-5084.
- Bradbury EJ, Burnstock G, McMahon SB (1998) The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Mol Cell Neurosci* 12:256-268.
- Brough D, Le Feuvre RA, Wheeler RD, Solovyova N, Hilfiker S, Rothwell NJ, Verkhatsky A (2003) Ca²⁺ stores and Ca²⁺ entry differentially contribute to the release of IL-1 beta and IL-1 alpha from murine macrophages. *Journal of immunology* 170:3029-3036.
- Brough D, Rothwell NJ (2007) Caspase-1-dependent processing of pro-interleukin-1beta is cytosolic and precedes cell death. *Journal of cell science* 120:772-781.
- Broz P, Newton K, Lamkanfi M, Mariathasan S, Dixit VM, Monack DM (2010) Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *The Journal of experimental medicine* 207:1745-1755.
- Bruey JM, Bruey-Sedano N, Newman R, Chandler S, Stehlik C, Reed JC (2004) PAN1/NALP2/PYPAF2, an inducible inflammatory mediator that regulates NF-kappaB and caspase-1 activation in macrophages. *J Biol Chem* 279:51897-51907.
- Bryan NB, Dorfleutner A, Rojanasakul Y, Stehlik C (2009) Activation of inflammasomes requires intracellular redistribution of the apoptotic speck-like protein containing a caspase recruitment domain. *Journal of immunology* 182:3173-3182.
- Bryant C, Fitzgerald KA (2009) Molecular mechanisms involved in inflammasome activation. *Trends Cell Biol* 19:455-464.
- Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. *Physiological reviews* 87:659-797.

- Cahill CM, Dray A,Coderre TJ (2003) Enhanced thermal antinociceptive potency and anti-allodynic effects of morphine following spinal administration of endotoxin. *Brain Res* 960:209-218.
- Calvo M, Dawes JM, Bennett DL (2012) The role of the immune system in the generation of neuropathic pain. *Lancet neurology* 11:629-642.
- Calvo M, Zhu N, Grist J, Ma Z, Loeb JA, Bennett DL (2011) Following nerve injury neuregulin-1 drives microglial proliferation and neuropathic pain via the MEK/ERK pathway. *Glia* 59:554-568.
- Calvo M, Zhu N, Tsantoulas C, Ma Z, Grist J, Loeb JA, Bennett DL (2010) Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury. *J Neurosci* 30:5437-5450.
- Campbell JN, Meyer RA (2006) Mechanisms of neuropathic pain. *Neuron* 52:77-92.
- Campbell JN, Raja SN, Meyer RA, Mackinnon SE (1988) Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain* 32:89-94.
- Cao L, DeLeo JA (2008) CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *European journal of immunology* 38:448-458.
- Cao L, Tanga FY, Deleo JA (2009) The contributing role of CD14 in toll-like receptor 4 dependent neuropathic pain. *Neuroscience* 158:896-903.
- Casals-Diaz L, Vivo M, Navarro X (2009) Nociceptive responses and spinal plastic changes of afferent C-fibers in three neuropathic pain models induced by sciatic nerve injury in the rat. *Experimental neurology* 217:84-95.
- Chakraborty S, Kaushik DK, Gupta M, Basu A (2010) Inflammasome signaling at the heart of central nervous system pathology. *J Neurosci Res* 88:1615-1631.
- Chan WY, Kohsaka S, Rezaie P (2007) The origin and cell lineage of microglia: new concepts. *Brain Res Rev* 53:344-354.
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *Journal of neuroscience methods* 53:55-63.
- Chattopadhyay S, Myers RR, Janes J, Shubayev V (2007) Cytokine regulation of MMP-9 in peripheral glia: implications for pathological processes and pain in injured nerve. *Brain, behavior, and immunity* 21:561-568.
- Chen CL, Broom DC, Liu Y, de Nooij JC, Li Z, Cen C, Samad OA, Jessell TM, Woolf CJ, Ma Q (2006) Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. *Neuron* 49:365-377.
- Chen G, Shaw MH, Kim YG, Nunez G (2009) NOD-like receptors: role in innate immunity and inflammatory disease. *Annual review of pathology* 4:365-398.

- Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CB, Casula MA, Yiangou Y, Birch R, Anand P, Buell GN (2005) Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain* 114:386-396.
- Chien LY, Cheng JK, Chu D, Cheng CF, Tsaur ML (2007) Reduced expression of A-type potassium channels in primary sensory neurons induces mechanical hypersensitivity. *J Neurosci* 27:9855-9865.
- Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, Basbaum AI, Chao MV, Julius D (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* 411:957-962.
- Clark AK, D'Aquisto F, Gentry C, Marchand F, McMahon SB, Malcangio M (2006) Rapid co-release of interleukin 1beta and caspase 1 in spinal cord inflammation. *J Neurochem* 99:868-880.
- Clark AK, Grist J, Al-Kashi A, Perretti M, Malcangio M (2012) Spinal cathepsin S and fractalkine contribute to chronic pain in the collagen-induced arthritis model. *Arthritis and rheumatism* 64:2038-2047.
- Clark AK, Staniland AA, Marchand F, Kaan TK, McMahon SB, Malcangio M (2010a) P2X7-dependent release of interleukin-1beta and nociception in the spinal cord following lipopolysaccharide. *J Neurosci* 30:573-582.
- Clark AK, Wodarski R, Guida F, Sasso O, Malcangio M (2010b) Cathepsin S release from primary cultured microglia is regulated by the P2X7 receptor. *Glia* 58:1710-1726.
- Clark AK, Yip PK, Grist J, Gentry C, Staniland AA, Marchand F, Dehvari M, Wotherspoon G, Winter J, Ullah J, Bevan S, Malcangio M (2007) Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. *Proc Natl Acad Sci U S A* 104:10655-10660.
- Clark AK, Yip PK, Malcangio M (2009) The liberation of fractalkine in the dorsal horn requires microglial cathepsin S. *J Neurosci* 29:6945-6954.
- Colburn RW, Rickman AJ, DeLeo JA (1999) The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. *Experimental neurology* 157:289-304.
- Coll RC, O'Neill LA (2011) The cytokine release inhibitory drug CRID3 targets ASC oligomerisation in the NLRP3 and AIM2 inflammasomes. *PloS one* 6:e29539.
- Collino M, Benetti E, Rogazzo M, Mastrocola R, Yaqoob MM, Aragno M, Thiernemann C, Fantozzi R (2012) Reversal of the deleterious effects of chronic dietary HFCS-55 intake by PPAR-delta agonism correlates with impaired NLRP3 inflammasome activation. *Biochemical pharmacology*.

- Costigan M, Moss A, Latremoliere A, Johnston C, Verma-Gandhu M, Herbert TA, Barrett L, Brenner GJ, Vardeh D, Woolf CJ, Fitzgerald M (2009a) T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. *J Neurosci* 29:14415-14422.
- Costigan M, Scholz J, Woolf CJ (2009b) Neuropathic pain: a maladaptive response of the nervous system to damage. *Annu Rev Neurosci* 32:1-32.
- Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y (2005) BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 438:1017-1021.
- Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sik A, De Koninck P, De Koninck Y (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* 424:938-942.
- Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG (2006) An SCN9A channelopathy causes congenital inability to experience pain. *Nature* 444:894-898.
- Cruz CM, Rinna A, Forman HJ, Ventura AL, Persechini PM, Ojcius DM (2007) ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 282:2871-2879.
- Cui JG, Holmin S, Mathiesen T, Meyerson BA, Linderöth B (2000) Possible role of inflammatory mediators in tactile hypersensitivity in rat models of mononeuropathy. *Pain* 88:239-248.
- Cuisset L, Jeru I, Dumont B, Fabre A, Cochet E, Le Bozec J, Delpech M, Amselem S, Touitou I, and the French Csg (2011) Mutations in the autoinflammatory cryopyrin-associated periodic syndrome gene: epidemiological study and lessons from eight years of genetic analysis in France. *Annals of the rheumatic diseases* 70:495-499.
- Cunha JM, Cunha FQ, Poole S, Ferreira SH (2000) Cytokine-mediated inflammatory hyperalgesia limited by interleukin-1 receptor antagonist. *Br J Pharmacol* 130:1418-1424.
- D'Mello R, Dickenson AH (2008) Spinal cord mechanisms of pain. *Br J Anaesth* 101:8-16.
- Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB (2005) ATP mediates rapid microglial response to local brain injury in vivo. *Nature neuroscience* 8:752-758.
- de Jong EK, Dijkstra IM, Hensens M, Brouwer N, van Amerongen M, Liem RS, Boddeke HW, Biber K (2005) Vesicle-mediated transport and release of CCL21 in endangered

- neurons: a possible explanation for microglia activation remote from a primary lesion. *J Neurosci* 25:7548-7557.
- De Leo JA, Tawfik VL, LaCroix-Fralish ML (2006) The tetrapartite synapse: path to CNS sensitization and chronic pain. *Pain* 122:17-21.
- de Rivero Vaccari JP, Lotocki G, Marcillo AE, Dietrich WD, Keane RW (2008) A molecular platform in neurons regulates inflammation after spinal cord injury. *J Neurosci* 28:3404-3414.
- Decosterd I, Woolf CJ (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* 87:149-158.
- Devor M (2006) Sodium channels and mechanisms of neuropathic pain. *J Pain* 7:S3-S12.
- Di Virgilio F (2007) Liaisons dangereuses: P2X(7) and the inflammasome. *Trends in pharmacological sciences* 28:465-472.
- Dib-Hajj SD, Fjell J, Cummins TR, Zheng Z, Fried K, LaMotte R, Black JA, Waxman SG (1999) Plasticity of sodium channel expression in DRG neurons in the chronic constriction injury model of neuropathic pain. *Pain* 83:591-600.
- Dinarello CA (2005) The many worlds of reducing interleukin-1. *Arthritis and rheumatism* 52:1960-1967.
- Dinarello CA (2009) Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology* 27:519-550.
- Dinarello CA (2010) IL-1: discoveries, controversies and future directions. *European journal of immunology* 40:599-606.
- Dinarello CA (2011) A clinical perspective of IL-1beta as the gatekeeper of inflammation. *European journal of immunology* 41:1203-1217.
- Dixon WJ (1980) Efficient analysis of experimental observations. *Annual review of pharmacology and toxicology* 20:441-462.
- Djouhri L, Koutsikou S, Fang X, McMullan S, Lawson SN (2006) Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. *J Neurosci* 26:1281-1292.
- Dostert C, Guarda G, Romero JF, Menu P, Gross O, Tardivel A, Suva ML, Stehle JC, Kopf M, Stamenkovic I, Corradin G, Tschopp J (2009) Malarial hemozoin is a Nalp3 inflammasome activating danger signal. *PloS one* 4:e6510.
- Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320:674-677.

- Dowdall T, Robinson I, Meert TF (2005) Comparison of five different rat models of peripheral nerve injury. *Pharmacology, biochemistry, and behavior* 80:93-108.
- Druilhe A, Srinivasula SM, Razmara M, Ahmad M, Alnemri ES (2001) Regulation of IL-1 β generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. *Cell Death Differ* 8:649-657.
- Dugandzija-Novakovic S, Koszowski AG, Levinson SR, Shrager P (1995) Clustering of Na⁺ channels and node of Ranvier formation in remyelinating axons. *J Neurosci* 15:492-503.
- Duncan JA, Bergstralh DT, Wang Y, Willingham SB, Ye Z, Zimmermann AG, Ting JP (2007) Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. *Proc Natl Acad Sci U S A* 104:8041-8046.
- Dworkin RH, O'Connor AB, Audette J, Baron R, Gourlay GK, Haanpaa ML, Kent JL, Krane EJ, Lebel AA, Levy RM, Mackey SC, Mayer J, Miaskowski C, Raja SN, Rice AS, Schmader KE, Stacey B, Stanos S, Treede RD, Turk DC, Walco GA, Wells CD (2010) Recommendations for the pharmacological management of neuropathic pain: an overview and literature update. *Mayo Clinic proceedings Mayo Clinic* 85:S3-14.
- Echeverry S, Shi XQ, Rivest S, Zhang J (2011) Peripheral nerve injury alters blood-spinal cord barrier functional and molecular integrity through a selective inflammatory pathway. *J Neurosci* 31:10819-10828.
- Echeverry S, Shi XQ, Zhang J (2008) Characterization of cell proliferation in rat spinal cord following peripheral nerve injury and the relationship with neuropathic pain. *Pain* 135:37-47.
- Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453:1122-1126.
- Ellebedy AH, Lupfer C, Ghoneim HE, DeBeauchamp J, Kanneganti TD, Webby RJ (2011) Inflammasome-independent role of the apoptosis-associated speck-like protein containing CARD (ASC) in the adjuvant effect of MF59. *Proc Natl Acad Sci U S A* 108:2927-2932.
- Emery EC, Young GT, Berrocoso EM, Chen L, McNaughton PA (2011) HCN2 ion channels play a central role in inflammatory and neuropathic pain. *Science* 333:1462-1466.
- Emery EC, Young GT, McNaughton PA (2012) HCN2 ion channels: an emerging role as the pacemakers of pain. *Trends in pharmacological sciences* 33:456-463.
- Ernsberger U (2009) Role of neurotrophin signalling in the differentiation of neurons from dorsal root ganglia and sympathetic ganglia. *Cell Tissue Res* 336:349-384.

- Faggioni R, Cattley RC, Guo J, Flores S, Brown H, Qi M, Yin S, Hill D, Scully S, Chen C, Brankow D, Lewis J, Baikalov C, Yamane H, Meng T, Martin F, Hu S, Boone T, Senaldi G (2001) IL-18-binding protein protects against lipopolysaccharide- induced lethality and prevents the development of Fas/Fas ligand-mediated models of liver disease in mice. *Journal of immunology* 167:5913-5920.
- Falchi M, Ferrara F, Gharib C, Dib B (2001) Hyperalgesic effect of intrathecally administered interleukin-1 in rats. *Drugs under experimental and clinical research* 27:97-101.
- Fantuzzi G, Reed DA, Dinarello CA (1999) IL-12-induced IFN-gamma is dependent on caspase-1 processing of the IL-18 precursor. *J Clin Invest* 104:761-767.
- Faustin B, Lartigue L, Bruey JM, Luciano F, Sergienko E, Bailly-Maitre B, Volkmann N, Hanein D, Rouiller I, Reed JC (2007) Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Molecular cell* 25:713-724.
- Feldmann J, Prieur AM, Quartier P, Berquin P, Certain S, Cortis E, Teillac-Hamel D, Fischer A, de Saint Basile G (2002) Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. *American journal of human genetics* 71:198-203.
- Fernandes-Alnemri T, Wu J, Yu JW, Datta P, Miller B, Jankowski W, Rosenberg S, Zhang J, Alnemri ES (2007) The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death Differ* 14:1590-1604.
- Fields HL, Bry J, Hentall I, Zorman G (1983) The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. *J Neurosci* 3:2545-2552.
- Fink SL, Cookson BT (2005) Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infection and immunity* 73:1907-1916.
- Fink SL, Cookson BT (2006) Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cellular microbiology* 8:1812-1825.
- Fleetwood-Walker SM, Quinn JP, Wallace C, Blackburn-Munro G, Kelly BG, Fiskerstrand CE, Nash AA, Dalziel RG (1999) Behavioural changes in the rat following infection with varicella-zoster virus. *The Journal of general virology* 80 (Pt 9):2433-2436.
- Flugel A, Hager G, Horvat A, Spitzer C, Singer GM, Graeber MB, Kreutzberg GW, Schwaiger FW (2001) Neuronal MCP-1 expression in response to remote nerve injury. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 21:69-76.

- Franchi L, Eigenbrod T, Nunez G (2009) Cutting edge: TNF- α mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *Journal of immunology* 183:792-796.
- Fu KY, Tan YH, Sung B, Mao J (2009) Peripheral formalin injection induces unique spinal cord microglial phenotypic changes. *Neurosci Lett* 449:234-239.
- Fukuoka H, Kawatani M, Hisamitsu T, Takeshige C (1994) Cutaneous hyperalgesia induced by peripheral injection of interleukin-1 β in the rat. *Brain Res* 657:133-140.
- Gabay E, Wolf G, Shavit Y, Yirmiya R, Tal M (2011) Chronic blockade of interleukin-1 (IL-1) prevents and attenuates neuropathic pain behavior and spontaneous ectopic neuronal activity following nerve injury. *European journal of pain* 15:242-248.
- Gainsford T, Willson TA, Metcalf D, Handman E, McFarlane C, Ng A, Nicola NA, Alexander WS, Hilton DJ (1996) Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. *Proc Natl Acad Sci U S A* 93:14564-14568.
- Gao X, Kim HK, Chung JM, Chung K (2007) Reactive oxygen species (ROS) are involved in enhancement of NMDA-receptor phosphorylation in animal models of pain. *Pain* 131:262-271.
- Gao YJ, Zhang L, Samad OA, Suter MR, Yasuhiko K, Xu ZZ, Park JY, Lind AL, Ma Q, Ji RR (2009) JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. *J Neurosci* 29:4096-4108.
- Garden GA, Moller T (2006) Microglia biology in health and disease. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* 1:127-137.
- Gasse P, Riteau N, Charron S, Girre S, Fick L, Petrilli V, Tschopp J, Lagente V, Quesniaux VF, Ryffel B, Couillin I (2009) Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. *Am J Respir Crit Care Med* 179:903-913.
- Gonzalez HL, Carmichael N, Dostrovsky JO, Charlton MP (2005) Evaluation of the time course of plasma extravasation in the skin by digital image analysis. *J Pain* 6:681-688.
- Gracie JA, Robertson SE, McInnes IB (2003) Interleukin-18. *Journal of leukocyte biology* 73:213-224.
- Gris D, Ye Z, Iocca HA, Wen H, Craven RR, Gris P, Huang M, Schneider M, Miller SD, Ting JP (2010) NLRP3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating Th1 and Th17 responses. *Journal of immunology* 185:974-981.

- Guarda G, Zenger M, Yazdi AS, Schroder K, Ferrero I, Menu P, Tardivel A, Mattmann C, Tschopp J (2011) Differential expression of NLRP3 among hematopoietic cells. *Journal of immunology* 186:2529-2534.
- Guma M, Ronacher L, Liu-Bryan R, Takai S, Karin M, Corr M (2009) Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. *Arthritis and rheumatism* 60:3642-3650.
- Hains BC, Waxman SG (2006) Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci* 26:4308-4317.
- Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, Fitzgerald KA, Latz E, Moore KJ, Golenbock DT (2008) The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9:857-865.
- Hanamsagar R, Torres V, Kielian T (2011) Inflammasome activation and IL-1beta/IL-18 processing are influenced by distinct pathways in microglia. *J Neurochem* 119:736-748.
- Hanisch UK (2002) Microglia as a source and target of cytokines. *Glia* 40:140-155.
- Hanisch UK, Kettenmann H (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature neuroscience* 10:1387-1394.
- Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32:77-88.
- Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, Streit WJ, Salafranca MN, Adhikari S, Thompson DA, Botti P, Bacon KB, Feng L (1998) Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc Natl Acad Sci U S A* 95:10896-10901.
- Hathway GJ, Vega-Avelaira D, Moss A, Ingram R, Fitzgerald M (2009) Brief, low frequency stimulation of rat peripheral C-fibres evokes prolonged microglial-induced central sensitization in adults but not in neonates. *Pain* 144:110-118.
- Hawkins PN, Lachmann HJ, Aganna E, McDermott MF (2004) Spectrum of clinical features in Muckle-Wells syndrome and response to anakinra. *Arthritis and rheumatism* 50:607-612.
- Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D (2006) The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nature neuroscience* 9:1512-1519.
- Heinricher MM, Tavares I, Leith JL, Lumb BM (2009) Descending control of nociception: Specificity, recruitment and plasticity. *Brain Res Rev* 60:214-225.

- Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD (2001) Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nature genetics* 29:301-305.
- Holtmann H, Enninga J, Kalble S, Thiefes A, Dorrie A, Broemer M, Winzen R, Wilhelm A, Ninomiya-Tsuji J, Matsumoto K, Resch K, Kracht M (2001) The MAPK kinase kinase TAK1 plays a central role in coupling the interleukin-1 receptor to both transcriptional and RNA-targeted mechanisms of gene regulation. *J Biol Chem* 276:3508-3516.
- Honda S, Sasaki Y, Ohsawa K, Imai Y, Nakamura Y, Inoue K, Kohsaka S (2001) Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors. *J Neurosci* 21:1975-1982.
- Honor P, Menning PM, Rogers SD, Nichols ML, Basbaum AI, Besson JM, Mantyh PW (1999) Spinal substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. *J Neurosci* 19:7670-7678.
- Honore P, Donnelly-Roberts D, Namovic M, Zhong C, Wade C, Chandran P, Zhu C, Carroll W, Perez-Medrano A, Iwakura Y, Jarvis MF (2009) The antihyperalgesic activity of a selective P2X7 receptor antagonist, A-839977, is lost in IL-1 α knockout mice. *Behavioural brain research* 204:77-81.
- Honore P, Rogers SD, Schwei MJ, Salak-Johnson JL, Luger NM, Sabino MC, Clohisy DR, Mantyh PW (2000) Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. *Neuroscience* 98:585-598.
- Honore P, Wade CL, Zhong C, Harris RR, Wu C, Ghayur T, Iwakura Y, Decker MW, Faltynek C, Sullivan J, Jarvis MF (2006) Interleukin-1 α gene-deficient mice show reduced nociceptive sensitivity in models of inflammatory and neuropathic pain but not post-operative pain. *Behavioural brain research* 167:355-364.
- Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9:847-856.
- Hu JH, Yang JP, Liu L, Li CF, Wang LN, Ji FH, Cheng H (2012) Involvement of CX3CR1 in bone cancer pain through the activation of microglia p38 MAPK pathway in the spinal cord. *Brain Res* 1465:1-9.
- Hu P, Bembrick AL, Keay KA, McLachlan EM (2007) Immune cell involvement in dorsal root ganglia and spinal cord after chronic constriction or transection of the rat sciatic nerve. *Brain, behavior, and immunity* 21:599-616.

- Hu P, McLachlan EM (2002) Macrophage and lymphocyte invasion of dorsal root ganglia after peripheral nerve lesions in the rat. *Neuroscience* 112:23-38.
- Huang W, Calvo, M., Krau, K., Olausen, H., Varga, A., Bathgate, G., Nagy, I., Okuse, K., Bennett, D.L.H., Rice, A.S.C. (2013) Ethologically relevant behavioural changes and peripheral nerve neuropathology in a rat model of HIV antiretroviral drug stavudine induced peripheral neuropathy *Pain Article in Press*.
- Hucho T, Levine JD (2007) Signaling pathways in sensitization: toward a nociceptor cell biology. *Neuron* 55:365-376.
- Hudson LJ, Bevan S, Wotherspoon G, Gentry C, Fox A, Winter J (2001) VR1 protein expression increases in undamaged DRG neurons after partial nerve injury. *Eur J Neurosci* 13:2105-2114.
- Hughes DI, Scott DT, Todd AJ, Riddell JS (2003) Lack of evidence for sprouting of Abeta afferents into the superficial laminae of the spinal cord dorsal horn after nerve section. *J Neurosci* 23:9491-9499.
- Humke EW, Shriver SK, Starovasnik MA, Fairbrother WJ, Dixit VM (2000) ICEBERG: a novel inhibitor of interleukin-1 β generation. *Cell* 103:99-111.
- Hunt SP, Mantyh PW (2001) The molecular dynamics of pain control. *Nat Rev Neurosci* 2:83-91.
- Ibi M, Matsuno K, Shiba D, Katsuyama M, Iwata K, Kakehi T, Nakagawa T, Sango K, Shirai Y, Yokoyama T, Kaneko S, Saito N, Yabe-Nishimura C (2008) Reactive oxygen species derived from NOX1/NADPH oxidase enhance inflammatory pain. *J Neurosci* 28:9486-9494.
- Inglis JJ, Nissim A, Lees DM, Hunt SP, Chernajovsky Y, Kidd BL (2005) The differential contribution of tumour necrosis factor to thermal and mechanical hyperalgesia during chronic inflammation. *Arthritis research & therapy* 7:R807-816.
- Inohara N, Koseki T, Lin J, del Peso L, Lucas PC, Chen FF, Ogura Y, Nunez G (2000) An induced proximity model for NF- κ B activation in the Nod1/RICK and RIP signaling pathways. *J Biol Chem* 275:27823-27831.
- Ippagunta SK, Brand DD, Luo J, Boyd KL, Calabrese C, Stienstra R, Van de Veerdonk FL, Netea MG, Joosten LA, Lamkanfi M, Kanneganti TD (2010) Inflammasome-independent role of apoptosis-associated speck-like protein containing a CARD (ASC) in T cell priming is critical for collagen-induced arthritis. *J Biol Chem* 285:12454-12462.
- Jaggi AS, Singh N (2012) Mechanisms in cancer-chemotherapeutic drugs-induced peripheral neuropathy. *Toxicology* 291:1-9.

- Janeway CA, Jr., Medzhitov R (2002) Innate immune recognition. *Annual review of immunology* 20:197-216.
- Ji RR, Kawasaki Y, Zhuang ZY, Wen YR, Decosterd I (2006) Possible role of spinal astrocytes in maintaining chronic pain sensitization: review of current evidence with focus on bFGF/JNK pathway. *Neuron glia biology* 2:259-269.
- Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* 36:57-68.
- Ji RR, Suter MR (2007) p38 MAPK, microglial signaling, and neuropathic pain. *Mol Pain* 3:33.
- Ji RR, Zhang Q, Zhang X, Piehl F, Reilly T, Pettersson RF, Hokfelt T (1995) Prominent expression of bFGF in dorsal root ganglia after axotomy. *Eur J Neurosci* 7:2458-2468.
- Jin C, Frayssinet P, Pelker R, Cwirka D, Hu B, Vignery A, Eisenbarth SC, Flavell RA (2011) NLRP3 inflammasome plays a critical role in the pathogenesis of hydroxyapatite-associated arthropathy. *Proc Natl Acad Sci U S A* 108:14867-14872.
- Jin SX, Zhuang ZY, Woolf CJ, Ji RR (2003) p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci* 23:4017-4022.
- Joseph EK, Levine JD (2004) Caspase signalling in neuropathic and inflammatory pain in the rat. *Eur J Neurosci* 20:2896-2902.
- Juliana C, Fernandes-Alnemri T, Wu J, Datta P, Solorzano L, Yu JW, Meng R, Quong AA, Latz E, Scott CP, Alnemri ES (2010) Anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome. *J Biol Chem* 285:9792-9802.
- Jura J, Wegrzyn P, Korostynski M, Guzik K, Oczko-Wojciechowska M, Jarzab M, Kowalska M, Piechota M, Przewlocki R, Koj A (2008) Identification of interleukin-1 and interleukin-6-responsive genes in human monocyte-derived macrophages using microarrays. *Biochimica et biophysica acta* 1779:383-389.
- Kahlenberg JM, Dubyak GR (2004) Mechanisms of caspase-1 activation by P2X7 receptor-mediated K⁺ release. *American journal of physiology Cell physiology* 286:C1100-1108.
- Kahlenberg JM, Lundberg KC, Kertesz SB, Qu Y, Dubyak GR (2005) Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF-kappaB-driven protein synthesis. *Journal of immunology* 175:7611-7622.
- Kavathas PB, Boeras CM, Mulla MJ, Abrahams VM (2012) Nod1, but not the ASC inflammasome, contributes to induction of IL-1beta secretion in human trophoblasts after sensing of *Chlamydia trachomatis*. *Mucosal immunology*.

- Kawanokuchi J, Mizuno T, Takeuchi H, Kato H, Wang J, Mitsuma N, Suzumura A (2006) Production of interferon-gamma by microglia. *Multiple sclerosis* 12:558-564.
- Kawasaki Y, Xu ZZ, Wang X, Park JY, Zhuang ZY, Tan PH, Gao YJ, Roy K, Corfas G, Lo EH, Ji RR (2008a) Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain. *Nat Med* 14:331-336.
- Kawasaki Y, Zhang L, Cheng JK, Ji RR (2008b) Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J Neurosci* 28:5189-5194.
- Keller M, Ruegg A, Werner S, Beer HD (2008) Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 132:818-831.
- Khare S, Luc N, Dorfleutner A, Stehlik C (2010) Inflammasomes and their activation. *Critical reviews in immunology* 30:463-487.
- Kidd BL, Urban LA (2001) Mechanisms of inflammatory pain. *Br J Anaesth* 87:3-11.
- Kiguchi N, Kobayashi Y, Kishioka S (2012) Chemokines and cytokines in neuroinflammation leading to neuropathic pain. *Curr Opin Pharmacol* 12:55-61.
- Kiguchi N, Maeda T, Kobayashi Y, Kishioka S (2008) Up-regulation of tumor necrosis factor-alpha in spinal cord contributes to vincristine-induced mechanical allodynia in mice. *Neurosci Lett* 445:140-143.
- Kim CF, Moalem-Taylor G (2011) Detailed characterization of neuro-immune responses following neuropathic injury in mice. *Brain Res* 1405:95-108.
- Kim D, You B, Jo EK, Han SK, Simon MI, Lee SJ (2010a) NADPH oxidase 2-derived reactive oxygen species in spinal cord microglia contribute to peripheral nerve injury-induced neuropathic pain. *Proc Natl Acad Sci U S A* 107:14851-14856.
- Kim DS, Figueroa KW, Li KW, Boroujerdi A, Yolo T, Luo ZD (2009) Profiling of dynamically changed gene expression in dorsal root ganglia post peripheral nerve injury and a critical role of injury-induced glial fibrillary acidic protein in maintenance of pain behaviors [corrected]. *Pain* 143:114-122.
- Kim KJ, Yoon YW, Chung JM (1997) Comparison of three rodent neuropathic pain models. *Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale* 113:200-206.
- Kim S, Bauernfeind F, Ablasser A, Hartmann G, Fitzgerald KA, Latz E, Hornung V (2010b) *Listeria monocytogenes* is sensed by the NLRP3 and AIM2 inflammasome. *European journal of immunology* 40:1545-1551.

- Kim SH, Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 50:355-363.
- Kingham PJ, Cuzner ML, Pocock JM (1999) Apoptotic pathways mobilized in microglia and neurones as a consequence of chromogranin A-induced microglial activation. *J Neurochem* 73:538-547.
- Kingham PJ, Pocock JM (2000) Microglial apoptosis induced by chromogranin A is mediated by mitochondrial depolarisation and the permeability transition but not by cytochrome c release. *J Neurochem* 74:1452-1462.
- Kobayashi H, Chattopadhyay S, Kato K, Dolkas J, Kikuchi S, Myers RR, Shubayev VI (2008a) MMPs initiate Schwann cell-mediated MBP degradation and mechanical nociception after nerve damage. *Mol Cell Neurosci* 39:619-627.
- Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA (2002) RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416:194-199.
- Kobayashi K, Yamanaka H, Fukuoka T, Dai Y, Obata K, Noguchi K (2008b) P2Y₁₂ receptor upregulation in activated microglia is a gateway of p38 signaling and neuropathic pain. *J Neurosci* 28:2892-2902.
- Kofuji P, Newman EA (2004) Potassium buffering in the central nervous system. *Neuroscience* 129:1045-1056.
- Kohama I, Ishikawa K, Kocsis JD (2000) Synaptic reorganization in the substantia gelatinosa after peripheral nerve neuroma formation: aberrant innervation of lamina II neurons by Abeta afferents. *J Neurosci* 20:1538-1549.
- Kolly L, Karababa M, Joosten LA, Narayan S, Salvi R, Petrilli V, Tschopp J, van den Berg WB, So AK, Busso N (2009) Inflammatory role of ASC in antigen-induced arthritis is independent of caspase-1, NALP-3, and IPAF. *Journal of immunology* 183:4003-4012.
- Korherr C, Hofmeister R, Wesche H, Falk W (1997) A critical role for interleukin-1 receptor accessory protein in interleukin-1 signaling. *European journal of immunology* 27:262-267.
- Kretschmer T, Happel LT, England JD, Nguyen DH, Tiel RL, Beuerman RW, Kline DG (2002) Accumulation of PN1 and PN3 sodium channels in painful human neuroma-evidence from immunocytochemistry. *Acta Neurochir (Wien)* 144:803-810; discussion 810.
- Kumar S, Boehm J, Lee JC (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov* 2:717-726.
- Kumar V, Sharma A (2010) Neutrophils: Cinderella of innate immune system. *International immunopharmacology* 10:1325-1334.

- Kummer JA, Broekhuizen R, Everett H, Agostini L, Kuijk L, Martinon F, van Bruggen R, Tschopp J (2007) Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. *J Histochem Cytochem* 55:443-452.
- Lamkanfi M, Denecker G, Kalai M, D'Hondt K, Meeus A, Declercq W, Saelens X, Vandenabeele P (2004) INCA, a novel human caspase recruitment domain protein that inhibits interleukin-1 β generation. *J Biol Chem* 279:51729-51738.
- Lamkanfi M, Dixit VM (2009) Inflammasomes: guardians of cytosolic sanctity. *Immunological reviews* 227:95-105.
- Lamkanfi M, Kanneganti TD, Franchi L, Nunez G (2007) Caspase-1 inflammasomes in infection and inflammation. *Journal of leukocyte biology* 82:220-225.
- Lamkanfi M, Mueller JL, Vitari AC, Misaghi S, Fedorova A, Deshayes K, Lee WP, Hoffman HM, Dixit VM (2009) Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *J Cell Biol* 187:61-70.
- Lara-Tejero M, Sutterwala FS, Ogura Y, Grant EP, Bertin J, Coyle AJ, Flavell RA, Galan JE (2006) Role of the caspase-1 inflammasome in *Salmonella typhimurium* pathogenesis. *The Journal of experimental medicine* 203:1407-1412.
- Latz E (2010) The inflammasomes: mechanisms of activation and function. *Curr Opin Immunol* 22:28-33.
- Lawson SN (2002) Phenotype and function of somatic primary afferent nociceptive neurones with C-, Delta- or Aalpha/beta-fibres. *Exp Physiol* 87:239-244.
- Le Feuvre RA, Brough D, Iwakura Y, Takeda K, Rothwell NJ (2002) Priming of macrophages with lipopolysaccharide potentiates P2X7-mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. *J Biol Chem* 277:3210-3218.
- Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, Watkins LR (2005) Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain* 115:71-83.
- Lee BH, Yoon YW, Chung K, Chung JM (1998) Comparison of sympathetic sprouting in sensory ganglia in three animal models of neuropathic pain. *Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale* 120:432-438.
- Lee DH, Chang L, Sorkin LS, Chaplan SR (2005) Hyperpolarization-activated, cation-nonselective, cyclic nucleotide-modulated channel blockade alleviates mechanical allodynia and suppresses ectopic discharge in spinal nerve ligated rats. *J Pain* 6:417-424.

- Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, Germain RN, Kastner DL, Chae JJ (2012) The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca^{2+} and cAMP. *Nature* 492:123-127.
- Lee HL, Lee KM, Son SJ, Hwang SH, Cho HJ (2004) Temporal expression of cytokines and their receptors mRNAs in a neuropathic pain model. *Neuroreport* 15:2807-2811.
- Lee KM, Jeon SM, Cho HJ (2010a) Interleukin-6 induces microglial CX3CR1 expression in the spinal cord after peripheral nerve injury through the activation of p38 MAPK. *European journal of pain* 14:682 e681-612.
- Lee S, Zhao YQ, Ribeiro-da-Silva A, Zhang J (2010b) Distinctive response of CNS glial cells in oro-facial pain associated with injury, infection and inflammation. *Mol Pain* 6:79.
- Leemans JC, Cassel SL, Sutterwala FS (2011) Sensing damage by the NLRP3 inflammasome. *Immunological reviews* 243:152-162.
- Leffler A, Cummins TR, Dib-Hajj SD, Hormuzdiar WN, Black JA, Waxman SG (2002) GDNF and NGF reverse changes in repriming of TTX-sensitive Na^{+} currents following axotomy of dorsal root ganglion neurons. *J Neurophysiol* 88:650-658.
- Leung L, Cahill CM (2010) TNF- α and neuropathic pain--a review. *Journal of neuroinflammation* 7:27.
- Levy BD, Clish CB, Schmidt B, Gronert K, Serhan CN (2001) Lipid mediator class switching during acute inflammation: signals in resolution. *Nat Immunol* 2:612-619.
- Li CY, Song YH, Higuera ES, Luo ZD (2004) Spinal dorsal horn calcium channel $\alpha_2\delta_1$ subunit upregulation contributes to peripheral nerve injury-induced tactile allodynia. *J Neurosci* 24:8494-8499.
- Li J, Hsu HC, Yang P, Wu Q, Li H, Edgington LE, Bogoy M, Kimberly RP, Mountz JD (2012) Treatment of arthritis by macrophage depletion and immunomodulation: testing an apoptosis-mediated therapy in a humanized death receptor mouse model. *Arthritis and rheumatism* 64:1098-1109.
- Li WW, Sabsovich I, Guo TZ, Zhao R, Kingery WS, Clark JD (2009) The role of enhanced cutaneous IL-1 β signaling in a rat tibia fracture model of complex regional pain syndrome. *Pain* 144:303-313.
- Liao YH, Zhang GH, Jia D, Wang P, Qian NS, He F, Zeng XT, He Y, Yang YL, Cao DY, Zhang Y, Wang DS, Tao KS, Gao CJ, Dou KF (2011) Spinal astrocytic activation contributes to mechanical allodynia in a mouse model of type 2 diabetes. *Brain Res* 1368:324-335.
- Lim G, Wang S, Zhang Y, Tian Y, Mao J (2009) Spinal leptin contributes to the pathogenesis of neuropathic pain in rodents. *J Clin Invest* 119:295-304.

- Lin T, Li K, Zhang FY, Zhang ZK, Light AR, Fu KY (2007) Dissociation of spinal microglia morphological activation and peripheral inflammation in inflammatory pain models. *Journal of neuroimmunology* 192:40-48.
- Liu T, van Rooijen N, Tracey DJ (2000a) Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury. *Pain* 86:25-32.
- Liu X, Eschenfelder S, Blenk KH, Janig W, Habler H (2000b) Spontaneous activity of axotomized afferent neurons after L5 spinal nerve injury in rats. *Pain* 84:309-318.
- Loiarro M, Ruggiero V, Sette C (2010) Targeting TLR/IL-1R signalling in human diseases. *Mediators of inflammation* 2010:674363.
- Lopez-Castejon G, Brough D (2011) Understanding the mechanism of IL-1 β secretion. *Cytokine & growth factor reviews* 22:189-195.
- Ma C, Shu Y, Zheng Z, Chen Y, Yao H, Greenquist KW, White FA, LaMotte RH (2003) Similar electrophysiological changes in axotomized and neighboring intact dorsal root ganglion neurons. *J Neurophysiol* 89:1588-1602.
- Ma W, Zhang Y, Bantel C, Eisenach JC (2005) Medium and large injured dorsal root ganglion cells increase TRPV-1, accompanied by increased α 2C-adrenoceptor co-expression and functional inhibition by clonidine. *Pain* 113:386-394.
- Maeda T, Kiguchi N, Kobayashi Y, Ikuta T, Ozaki M, Kishioka S (2009) Leptin derived from adipocytes in injured peripheral nerves facilitates development of neuropathic pain via macrophage stimulation. *Proc Natl Acad Sci U S A* 106:13076-13081.
- Maier SF, Wiertelak EP, Martin D, Watkins LR (1993) Interleukin-1 mediates the behavioral hyperalgesia produced by lithium chloride and endotoxin. *Brain Res* 623:321-324.
- Malcangio M, Ramer MS, Jones MG, McMahon SB (2000) Abnormal substance P release from the spinal cord following injury to primary sensory neurons. *Eur J Neurosci* 12:397-399.
- Malcangio M, Tomlinson DR (1998) A pharmacologic analysis of mechanical hyperalgesia in streptozotocin/diabetic rats. *Pain* 76:151-157.
- Malmberg AB, Chen C, Tonegawa S, Basbaum AI (1997) Preserved acute pain and reduced neuropathic pain in mice lacking PKC γ . *Science* 278:279-283.
- Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA (1997) Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science* 278:275-279.
- Maratou K, Wallace VC, Hasnie FS, Okuse K, Hosseini R, Jina N, Blackbeard J, Pheby T, Orengo C, Dickenson AH, McMahon SB, Rice AS (2009) Comparison of dorsal root ganglion

- gene expression in rat models of traumatic and HIV-associated neuropathic pain. *European journal of pain* 13:387-398.
- Marchand F, Perretti M, McMahon SB (2005) Role of the immune system in chronic pain. *Nat Rev Neurosci* 6:521-532.
- Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213-218.
- Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.
- Martinon F, Burns K, Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular cell* 10:417-426.
- Martinon F, Gaide O, Petrilli V, Mayor A, Tschopp J (2007) NALP inflammasomes: a central role in innate immunity. *Semin Immunopathol* 29:213-229.
- Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: guardians of the body. *Annual review of immunology* 27:229-265.
- Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237-241.
- Martinon F, Tschopp J (2004) Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 117:561-574.
- Martinon F, Tschopp J (2005) NLRs join TLRs as innate sensors of pathogens. *Trends Immunol* 26:447-454.
- Martinon F, Tschopp J (2007) Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ* 14:10-22.
- Masumoto J, Taniguchi S, Ayukawa K, Sarvotham H, Kishino T, Niikawa N, Hidaka E, Katsuyama T, Higuchi T, Sagara J (1999) ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *J Biol Chem* 274:33835-33838.
- Matarese G, Moschos S, Mantzoros CS (2005) Leptin in immunology. *Journal of immunology* 174:3137-3142.
- Matsui T, Svensson CI, Hirata Y, Mizobata K, Hua XY, Yaksh TL (2010) Release of prostaglandin E(2) and nitric oxide from spinal microglia is dependent on activation of p38 mitogen-activated protein kinase. *Anesth Analg* 111:554-560.

- Matsushita K, Takeoka M, Sagara J, Itano N, Kurose Y, Nakamura A, Taniguchi S (2009) A splice variant of ASC regulates IL-1 β release and aggregates differently from intact ASC. *Mediators of inflammation* 2009:287387.
- May A (2008) Chronic pain may change the structure of the brain. *Pain* 137:7-15.
- Mayor A, Martinon F, De Smedt T, Petrilli V, Tschopp J (2007) A crucial function of SGT1 and HSP90 in inflammasome activity links mammalian and plant innate immune responses. *Nat Immunol* 8:497-503.
- McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ, Maki RA (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO journal* 15:5647-5658.
- McMahon SB, Armanini MP, Ling LH, Phillips HS (1994) Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* 12:1161-1171.
- McMahon SB, Malcangio M (2009) Current challenges in glia-pain biology. *Neuron* 64:46-54.
- Medzhitov R (2008) Origin and physiological roles of inflammation. *Nature* 454:428-435.
- Medzhitov R, Janeway C, Jr. (2000) Innate immune recognition: mechanisms and pathways. *Immunological reviews* 173:89-97.
- Meller ST, Gebhart GF (1993) Nitric oxide (NO) and nociceptive processing in the spinal cord. *Pain* 52:127-136.
- Meller ST, Pechman PS, Gebhart GF, Maves TJ (1992) Nitric oxide mediates the thermal hyperalgesia produced in a model of neuropathic pain in the rat. *Neuroscience* 50:7-10.
- Mertens M, Singh JA (2009) Anakinra for rheumatoid arthritis: a systematic review. *The Journal of rheumatology* 36:1118-1125.
- Mestre C, Pelissier T, Fialip J, Wilcox G, Eschali r A (1994) A method to perform direct transcutaneous intrathecal injection in rats. *Journal of pharmacological and toxicological methods* 32:197-200.
- Miletic G, Miletic V (2008) Loose ligation of the sciatic nerve is associated with TrkB receptor-dependent decreases in KCC2 protein levels in the ipsilateral spinal dorsal horn. *Pain* 137:532-539.
- Millan MJ (1999) The induction of pain: an integrative review. *Prog Neurobiol* 57:1-164.
- Millan MJ (2002) Descending control of pain. *Prog Neurobiol* 66:355-474.
- Miller AV, Ranatunga SK (2012) Immunotherapies in rheumatologic disorders. *The Medical clinics of North America* 96:475-496, ix-x.

- Milligan ED, O'Connor KA, Nguyen KT, Armstrong CB, Twining C, Gaykema RP, Holguin A, Martin D, Maier SF, Watkins LR (2001) Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. *J Neurosci* 21:2808-2819.
- Miraucourt LS, Dallel R, Voisin DL (2007) Glycine inhibitory dysfunction turns touch into pain through PKCgamma interneurons. *PloS one* 2:e1116.
- Miyoshi K, Obata K, Kondo T, Okamura H, Noguchi K (2008) Interleukin-18-mediated microglia/astrocyte interaction in the spinal cord enhances neuropathic pain processing after nerve injury. *J Neurosci* 28:12775-12787.
- Moalem G, Tracey DJ (2006) Immune and inflammatory mechanisms in neuropathic pain. *Brain Res Rev* 51:240-264.
- Moalem G, Xu K, Yu L (2004) T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. *Neuroscience* 129:767-777.
- Molliver DC, Snider WD (1997) Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. *J Comp Neurol* 381:428-438.
- Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J Neurosci* 22:6724-6731.
- Mu X, Silos-Santiago I, Carroll SL, Snider WD (1993) Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. *J Neurosci* 13:4029-4041.
- Munder M, Mallo M, Eichmann K, Modolell M (1998) Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. *The Journal of experimental medicine* 187:2103-2108.
- Nadeau S, Filali M, Zhang J, Kerr BJ, Rivest S, Soulet D, Iwakura Y, de Rivero Vaccari JP, Keane RW, Lacroix S (2011) Functional recovery after peripheral nerve injury is dependent on the pro-inflammatory cytokines IL-1beta and TNF: implications for neuropathic pain. *J Neurosci* 31:12533-12542.
- Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, Englert JA, Rabinovitch M, Cernadas M, Kim HP, Fitzgerald KA, Ryter SW, Choi AM (2011) Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* 12:222-230.

- Nanki T, Urasaki Y, Imai T, Nishimura M, Muramoto K, Kubota T, Miyasaka N (2004) Inhibition of fractalkine ameliorates murine collagen-induced arthritis. *Journal of immunology* 173:7010-7016.
- Napoli I, Noon LA, Ribeiro S, Kerai AP, Parrinello S, Rosenberg LH, Collins MJ, Harrisingh MC, White IJ, Woodhoo A, Lloyd AC (2012) A central role for the ERK-signaling pathway in controlling Schwann cell plasticity and peripheral nerve regeneration in vivo. *Neuron* 73:729-742.
- Nathan CF (1987) Secretory products of macrophages. *J Clin Invest* 79:319-326.
- Navarro X, Vivo M, Valero-Cabre A (2007) Neural plasticity after peripheral nerve injury and regeneration. *Prog Neurobiol* 82:163-201.
- Neumann H, Kotter MR, Franklin RJ (2009) Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain* 132:288-295.
- Neumann S, Braz JM, Skinner K, Llewellyn-Smith IJ, Basbaum AI (2008) Innocuous, not noxious, input activates PKCgamma interneurons of the spinal dorsal horn via myelinated afferent fibers. *J Neurosci* 28:7936-7944.
- Neumann S, Doubell TP, Leslie T, Woolf CJ (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. *Nature* 384:360-364.
- Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308:1314-1318.
- Noguchi K, Dubner R, De Leon M, Senba E, Ruda MA (1994) Axotomy induces preprotachykinin gene expression in a subpopulation of dorsal root ganglion neurons. *J Neurosci Res* 37:596-603.
- Noguchi K, Senba E, Morita Y, Sato M, Tohyama M (1990) Alpha-CGRP and beta-CGRP mRNAs are differentially regulated in the rat spinal cord and dorsal root ganglion. *Brain Res Mol Brain Res* 7:299-304.
- Noma N, Khan J, Chen IF, Markman S, Benoliel R, Hadlaq E, Imamura Y, Eliav E (2011) Interleukin-17 levels in rat models of nerve damage and neuropathic pain. *Neurosci Lett* 493:86-91.
- Novakovic SD, Tzoumaka E, McGivern JG, Haraguchi M, Sangameswaran L, Gogas KR, Eglen RM, Hunter JC (1998) Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. *J Neurosci* 18:2174-2187.
- O'Connor W, Jr., Harton JA, Zhu X, Linhoff MW, Ting JP (2003) Cutting edge: CIAS1/cryopyrin/PYPAF1/NALP3/CATERPILLER 1.1 is an inducible inflammatory

- mediator with NF-kappa B suppressive properties. *Journal of immunology* 171:6329-6333.
- Ogura Y, Sutterwala FS, Flavell RA (2006) The inflammasome: first line of the immune response to cell stress. *Cell* 126:659-662.
- Oka T, Aou S, Hori T (1993) Intracerebroventricular injection of interleukin-1 beta induces hyperalgesia in rats. *Brain Res* 624:61-68.
- Oka T, Aou S, Hori T (1994) Intracerebroventricular injection of interleukin-1 beta enhances nociceptive neuronal responses of the trigeminal nucleus caudalis in rats. *Brain Res* 656:236-244.
- Okamoto K, Martin DP, Schmelzer JD, Mitsui Y, Low PA (2001) Pro- and anti-inflammatory cytokine gene expression in rat sciatic nerve chronic constriction injury model of neuropathic pain. *Experimental neurology* 169:386-391.
- Okun A, DeFelice M, Eyde N, Ren J, Mercado R, King T, Porreca F (2011) Transient inflammation-induced ongoing pain is driven by TRPV1 sensitive afferents. *Mol Pain* 7:4.
- Oosting M, Buffen K, Malireddi SR, Sturm P, Verschueren I, Koenders MI, van de Veerdonk FL, van der Meer JW, Netea MG, Kanneganti TD, Joosten LA (2012) Murine *Borrelia* arthritis is highly dependent on ASC and caspase-1, but independent of NLRP3. *Arthritis research & therapy* 14:R247.
- Ozaktay AC, Kallakuri S, Takebayashi T, Cavanaugh JM, Asik I, DeLeo JA, Weinstein JN (2006) Effects of interleukin-1 beta, interleukin-6, and tumor necrosis factor on sensitivity of dorsal root ganglion and peripheral receptive fields in rats. *European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society* 15:1529-1537.
- Palmer MT, Weaver CT (2010) Autoimmunity: increasing suspects in the CD4+ T cell lineup. *Nat Immunol* 11:36-40.
- Parks WC, Wilson CL, Lopez-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nature reviews Immunology* 4:617-629.
- Paulukat J, Bosmann M, Nold M, Garkisch S, Kampfer H, Frank S, Raedle J, Zeuzem S, Pfeilschifter J, Muhl H (2001) Expression and release of IL-18 binding protein in response to IFN-gamma. *Journal of immunology* 167:7038-7043.
- Pekny M, Nilsson M (2005) Astrocyte activation and reactive gliosis. *Glia* 50:427-434.

- Pelegrin P, Barroso-Gutierrez C, Surprenant A (2008) P2X7 receptor differentially couples to distinct release pathways for IL-1 β in mouse macrophage. *Journal of immunology* 180:7147-7157.
- Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1 β release by the ATP-gated P2X7 receptor. *The EMBO journal* 25:5071-5082.
- Perkins NM, Tracey DJ (2000) Hyperalgesia due to nerve injury: role of neutrophils. *Neuroscience* 101:745-757.
- Perregaux D, Gabel CA (1994) Interleukin-1 β maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem* 269:15195-15203.
- Petrilli V, Dostert C, Muruve DA, Tschopp J (2007a) The inflammasome: a danger sensing complex triggering innate immunity. *Curr Opin Immunol* 19:615-622.
- Petrilli V, Martinon F (2007) The inflammasome, autoinflammatory diseases, and gout. *Joint Bone Spine* 74:571-576.
- Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J (2007b) Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14:1583-1589.
- Petrilli V, Papin S, Tschopp J (2005) The inflammasome. *Curr Biol* 15:R581.
- Pezet S, McMahon SB (2006) Neurotrophins: mediators and modulators of pain. *Annu Rev Neurosci* 29:507-538.
- Pintaux E, Inoue W, Schmidt L, Molina-Holgado F, Rothwell NJ, Luheshi GN (2007) Leptin induces interleukin-1 β release from rat microglial cells through a caspase 1 independent mechanism. *J Neurochem* 102:826-833.
- Polgar E, Fowler JH, McGill MM, Todd AJ (1999) The types of neuron which contain protein kinase C γ in rat spinal cord. *Brain Res* 833:71-80.
- Polgar E, Hughes DI, Arham AZ, Todd AJ (2005) Loss of neurons from laminae I-III of the spinal dorsal horn is not required for development of tactile allodynia in the spared nerve injury model of neuropathic pain. *J Neurosci* 25:6658-6666.
- Porreca F, Ossipov MH, Gebhart GF (2002) Chronic pain and medullary descending facilitation. *Trends in neurosciences* 25:319-325.
- Qu Y, Franchi L, Nunez G, Dubyak GR (2007) Nonclassical IL-1 β secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *Journal of immunology* 179:1913-1925.
- Qu Y, Ramachandra L, Mohr S, Franchi L, Harding CV, Nunez G, Dubyak GR (2009) P2X7 receptor-stimulated secretion of MHC class II-containing exosomes requires the

- ASC/NLRP3 inflammasome but is independent of caspase-1. *Journal of immunology* 182:5052-5062.
- Rabuffetti M, Sciorati C, Tarozzo G, Clementi E, Manfredi AA, Beltramo M (2000) Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. *J Neurosci* 20:4398-4404.
- Raghavendra V, Tanga F, DeLeo JA (2003) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *The Journal of pharmacology and experimental therapeutics* 306:624-630.
- Raghavendra V, Tanga FY, DeLeo JA (2004) Complete Freund's adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. *Eur J Neurosci* 20:467-473.
- Rahman W, D'Mello R, Dickenson AH (2008) Peripheral nerve injury-induced changes in spinal $\alpha(2)$ -adrenoceptor-mediated modulation of mechanically evoked dorsal horn neuronal responses. *J Pain* 9:350-359.
- Ransohoff RM, Perry VH (2009) Microglial physiology: unique stimuli, specialized responses. *Annual review of immunology* 27:119-145.
- Rappert A, Biber K, Nolte C, Lipp M, Schubel A, Lu B, Gerard NP, Gerard C, Boddeke HW, Kettenmann H (2002) Secondary lymphoid tissue chemokine (CCL21) activates CXCR3 to trigger a Cl^- current and chemotaxis in murine microglia. *Journal of immunology* 168:3221-3226.
- Raupach B, Peuschel SK, Monack DM, Zychlinsky A (2006) Caspase-1-mediated activation of interleukin-1 β (IL-1 β) and IL-18 contributes to innate immune defenses against *Salmonella enterica* serovar Typhimurium infection. *Infection and immunity* 74:4922-4926.
- Ray K (2011) Crystal arthritis: NLRP3 inflammasome mediates crystal-induced joint inflammation and dysfunction. *Nature reviews Rheumatology* 7:684.
- Ren K, Torres R (2009) Role of interleukin-1 β during pain and inflammation. *Brain Res Rev* 60:57-64.
- Ridet JL, Malhotra SK, Privat A, Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. *Trends in neurosciences* 20:570-577.
- Romero-Sandoval A, Chai N, Natile-McMenemy N, DeLeo JA (2008) A comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. *Brain Res* 1219:116-126.

- Rosengren S, Hoffman HM, Bugbee W, Boyle DL (2005) Expression and regulation of cryopyrin and related proteins in rheumatoid arthritis synovium. *Annals of the rheumatic diseases* 64:708-714.
- Rossol M, Pierer M, Raulien N, Quandt D, Meusch U, Rothe K, Schubert K, Schoneberg T, Schaefer M, Krugel U, Smajilovic S, Brauner-Osborne H, Baerwald C, Wagner U (2012) Extracellular Ca(2+) is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nature communications* 3:1329.
- Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ (1995) Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. *Br J Pharmacol* 115:1265-1275.
- Saleh M, Mathison JC, Wolinski MK, Bensinger SJ, Fitzgerald P, Droin N, Ulevitch RJ, Green DR, Nicholson DW (2006) Enhanced bacterial clearance and sepsis resistance in caspase-12-deficient mice. *Nature* 440:1064-1068.
- Salvemini D, Little JW, Doyle T, Neumann WL (2011) Roles of reactive oxygen and nitrogen species in pain. *Free radical biology & medicine* 51:951-966.
- Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, Woolf CJ (2001) Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 410:471-475.
- Samad TA, Sapirstein A, Woolf CJ (2002) Prostanoids and pain: unraveling mechanisms and revealing therapeutic targets. *Trends in molecular medicine* 8:390-396.
- Sanz JM, Di Virgilio F (2000) Kinetics and mechanism of ATP-dependent IL-1 beta release from microglial cells. *Journal of immunology* 164:4893-4898.
- Schafers M, Brinkhoff J, Neukirchen S, Marziniak M, Sommer C (2001) Combined epineurial therapy with neutralizing antibodies to tumor necrosis factor-alpha and interleukin-1 receptor has an additive effect in reducing neuropathic pain in mice. *Neurosci Lett* 310:113-116.
- Schaible HG, Richter F, Ebersberger A, Boettger MK, Vanegas H, Natura G, Vazquez E, Segond von Banchet G (2009) Joint pain. *Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale* 196:153-162.
- Schaible HG, von Banchet GS, Boettger MK, Brauer R, Gajda M, Richter F, Hensellek S, Brenn D, Natura G (2010) The role of proinflammatory cytokines in the generation and maintenance of joint pain. *Ann N Y Acad Sci* 1193:60-69.
- Schmidt R, Schmelz M, Forster C, Ringkamp M, Torebjork E, Handwerker H (1995) Novel classes of responsive and unresponsive C nociceptors in human skin. *J Neurosci* 15:333-341.

- Schoeniger-Skinner DK, Ledebor A, Frank MG, Milligan ED, Poole S, Martin D, Maier SF, Watkins LR (2007) Interleukin-6 mediates low-threshold mechanical allodynia induced by intrathecal HIV-1 envelope glycoprotein gp120. *Brain, behavior, and immunity* 21:660-667.
- Scholz J, Abele A, Marian C, Haussler A, Herbert TA, Woolf CJ, Tegeder I (2008) Low-dose methotrexate reduces peripheral nerve injury-evoked spinal microglial activation and neuropathic pain behavior in rats. *Pain* 138:130-142.
- Scholz J, Broom DC, Youn DH, Mills CD, Kohno T, Suter MR, Moore KA, Decosterd I, Coggeshall RE, Woolf CJ (2005) Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury. *J Neurosci* 25:7317-7323.
- Schomberg D, Olson JK (2012) Immune responses of microglia in the spinal cord: contribution to pain states. *Experimental neurology* 234:262-270.
- Schonbeck U, Mach F, Libby P (1998) Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *Journal of immunology* 161:3340-3346.
- Schroder K, Tschopp J (2010) The inflammasomes. *Cell* 140:821-832.
- Schwei MJ, Honore P, Rogers SD, Salak-Johnson JL, Finke MP, Ramnaraine ML, Clohisey DR, Mantyh PW (1999) Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. *J Neurosci* 19:10886-10897.
- Segond von Banchet G, Boettger MK, Fischer N, Gajda M, Brauer R, Schaible HG (2009) Experimental arthritis causes tumor necrosis factor-alpha-dependent infiltration of macrophages into rat dorsal root ganglia which correlates with pain-related behavior. *Pain* 145:151-159.
- Seltzer Z, Dubner R, Shir Y (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* 43:205-218.
- Serhan CN, Chiang N, Van Dyke TE (2008) Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature reviews Immunology* 8:349-361.
- Serhan CN, Savill J (2005) Resolution of inflammation: the beginning programs the end. *Nat Immunol* 6:1191-1197.
- Shaw PJ, Lukens JR, Burns S, Chi H, McGargill MA, Kanneganti TD (2010) Cutting edge: critical role for PYCARD/ASC in the development of experimental autoimmune encephalomyelitis. *Journal of immunology* 184:4610-4614.

- Shehab SA, Spike RC, Todd AJ (2003) Evidence against cholera toxin B subunit as a reliable tracer for sprouting of primary afferents following peripheral nerve injury. *Brain Res* 964:218-227.
- Shen A, Zhou D, Shen Q, Liu HO, Sun L, Liu Y, Chen J, Yang J, Ji Y, Cheng C (2009) The expression of tumor necrosis factor-alpha (TNF-alpha) by the intrathecal injection of lipopolysaccharide in the rat spinal cord. *Neurochemical research* 34:333-341.
- Shields SD, Eckert WA, 3rd, Basbaum AI (2003) Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. *J Pain* 4:465-470.
- Shinkai K, McCalmont TH, Leslie KS (2008) Cryopyrin-associated periodic syndromes and autoinflammation. *Clinical and experimental dermatology* 33:1-9.
- Shubayev VI, Angert M, Dolkas J, Campana WM, Palenscar K, Myers RR (2006) TNFalpha-induced MMP-9 promotes macrophage recruitment into injured peripheral nerve. *Mol Cell Neurosci* 31:407-415.
- Smith FM, Haskelberg H, Tracey DJ, Moalem-Taylor G (2007) Role of histamine H3 and H4 receptors in mechanical hyperalgesia following peripheral nerve injury. *Neuroimmunomodulation* 14:317-325.
- Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron* 20:629-632.
- Sommer C, Kress M (2004) Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neurosci Lett* 361:184-187.
- Sommer C, Petrusch S, Lindenlaub T, Toyka KV (1999) Neutralizing antibodies to interleukin 1-receptor reduce pain associated behavior in mice with experimental neuropathy. *Neurosci Lett* 270:25-28.
- Srinivasula SM, Poyet JL, Razmara M, Datta P, Zhang Z, Alnemri ES (2002) The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. *J Biol Chem* 277:21119-21122.
- Staniland AA, Clark AK, Wodarski R, Sasso O, Maione F, D'Acquisto F, Malcangio M (2010) Reduced inflammatory and neuropathic pain and decreased spinal microglial response in fractalkine receptor (CX3CR1) knockout mice. *J Neurochem* 114:1143-1157.
- Stehlik C, Dorfleutner A (2007) COPs and POPs: modulators of inflammasome activity. *Journal of immunology* 179:7993-7998.
- Stehlik C, Fiorentino L, Dorfleutner A, Bruey JM, Ariza EM, Sagara J, Reed JC (2002) The PAAD/PYRIN-family protein ASC is a dual regulator of a conserved step in nuclear

- factor kappaB activation pathways. *The Journal of experimental medicine* 196:1605-1615.
- Stucky CL, Lewin GR (1999) Isolectin B(4)-positive and -negative nociceptors are functionally distinct. *J Neurosci* 19:6497-6505.
- Stych B, Dobrovolny D (2008) Familial cold auto-inflammatory syndrome (FCAS): characterization of symptomatology and impact on patients' lives. *Current medical research and opinion* 24:1577-1582.
- Subang MC, Richardson PM (2001) Influence of injury and cytokines on synthesis of monocyte chemoattractant protein-1 mRNA in peripheral nervous tissue. *Eur J Neurosci* 13:521-528.
- Subramaniam S, Stansberg C, Cunningham C (2004) The interleukin 1 receptor family. *Developmental and comparative immunology* 28:415-428.
- Sun T, Yu E, Yu L, Luo J, Li H, Fu Z (2012a) LipoxinA(4) induced antinociception and decreased expression of NF-kappaB and pro-inflammatory cytokines after chronic dorsal root ganglia compression in rats. *European journal of pain* 16:18-27.
- Sun YE, Peng L, Sun X, Bo J, Yang D, Zheng Y, Liu C, Zhu B, Ma Z, Gu X (2012b) Intrathecal injection of spironolactone attenuates radicular pain by inhibition of spinal microglia activation in a rat model. *PloS one* 7:e39897.
- Sung CS, Wen ZH, Chang WK, Chan KH, Ho ST, Tsai SK, Chang YC, Wong CS (2005) Inhibition of p38 mitogen-activated protein kinase attenuates interleukin-1beta-induced thermal hyperalgesia and inducible nitric oxide synthase expression in the spinal cord. *J Neurochem* 94:742-752.
- Sung CS, Wen ZH, Chang WK, Ho ST, Tsai SK, Chang YC, Wong CS (2004) Intrathecal interleukin-1beta administration induces thermal hyperalgesia by activating inducible nitric oxide synthase expression in the rat spinal cord. *Brain Res* 1015:145-153.
- Suter MR, Berta T, Gao YJ, Decosterd I, Ji RR (2009) Large A-fiber activity is required for microglial proliferation and p38 MAPK activation in the spinal cord: different effects of resiniferatoxin and bupivacaine on spinal microglial changes after spared nerve injury. *Mol Pain* 5:53.
- Sutterwala FS, Ogura Y, Szczepanik M, Lara-Tejero M, Lichtenberger GS, Grant EP, Bertin J, Coyle AJ, Galan JE, Askenase PW, Flavell RA (2006) Critical role for NALP3/CIA1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 24:317-327.

- Suzuki R, Morcuende S, Webber M, Hunt SP, Dickenson AH (2002) Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. *Nature neuroscience* 5:1319-1326.
- Suzuki T, Hide I, Ido K, Kohsaka S, Inoue K, Nakata Y (2004) Production and release of neuroprotective tumor necrosis factor by P2X7 receptor-activated microglia. *J Neurosci* 24:1-7.
- Svensson CI, Marsala M, Westerlund A, Calcutt NA, Campana WM, Freshwater JD, Catalano R, Feng Y, Protter AA, Scott B, Yaksh TL (2003) Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. *J Neurochem* 86:1534-1544.
- Sweitzer S, Martin D, DeLeo JA (2001) Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. *Neuroscience* 103:529-539.
- Sweitzer SM, Colburn RW, Rutkowski M, DeLeo JA (1999) Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behavior in the rat. *Brain Res* 829:209-221.
- Sweitzer SM, Pahl JL, DeLeo JA (2006) Propentofylline attenuates vincristine-induced peripheral neuropathy in the rat. *Neurosci Lett* 400:258-261.
- Takahashi K, Rochford CD, Neumann H (2005) Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *The Journal of experimental medicine* 201:647-657.
- Takeda M, Kitagawa J, Takahashi M, Matsumoto S (2008) Activation of interleukin-1beta receptor suppresses the voltage-gated potassium currents in the small-diameter trigeminal ganglion neurons following peripheral inflammation. *Pain* 139:594-602.
- Takeda M, Tanimoto T, Ikeda M, Nasu M, Kadoi J, Yoshida S, Matsumoto S (2006) Enhanced excitability of rat trigeminal root ganglion neurons via decrease in A-type potassium currents following temporomandibular joint inflammation. *Neuroscience* 138:621-630.
- Talbot S, Chahmi E, Dias JP, Couture R (2010) Key role for spinal dorsal horn microglial kinin B1 receptor in early diabetic pain neuropathy. *Journal of neuroinflammation* 7:36.
- Tanaka D, Kagari T, Doi H, Shimozato T (2006) Essential role of neutrophils in anti-type II collagen antibody and lipopolysaccharide-induced arthritis. *Immunology* 119:195-202.

- Tanaka M, Cummins TR, Ishikawa K, Dib-Hajj SD, Black JA, Waxman SG (1998) SNS Na⁺ channel expression increases in dorsal root ganglion neurons in the carrageenan inflammatory pain model. *Neuroreport* 9:967-972.
- Tanaka T, Minami M, Nakagawa T, Satoh M (2004) Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain: possible involvement in the development of neuropathic pain. *Neuroscience research* 48:463-469.
- Tanga FY, Nutile-McMenemy N, DeLeo JA (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* 102:5856-5861.
- Tanga FY, Raghavendra V, DeLeo JA (2004) Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochemistry international* 45:397-407.
- Tanga FY, Raghavendra V, Nutile-McMenemy N, Marks A, Deleo JA (2006) Role of astrocytic S100beta in behavioral hypersensitivity in rodent models of neuropathic pain. *Neuroscience* 140:1003-1010.
- Tate S, Benn S, Hick C, Trezise D, John V, Mannion RJ, Costigan M, Plumpton C, Grose D, Gladwell Z, Kendall G, Dale K, Bountra C, Woolf CJ (1998) Two sodium channels contribute to the TTX-R sodium current in primary sensory neurons. *Nature neuroscience* 1:653-655.
- Taxman DJ, Holley-Guthrie EA, Huang MT, Moore CB, Bergstralh DT, Allen IC, Lei Y, Gris D, Ting JP (2011) The NLR adaptor ASC/PYCARD regulates DUSP10, mitogen-activated protein kinase (MAPK), and chemokine induction independent of the inflammasome. *J Biol Chem* 286:19605-19616.
- Thacker MA, Clark AK, Bishop T, Grist J, Yip PK, Moon LD, Thompson SW, Marchand F, McMahon SB (2009) CCL2 is a key mediator of microglia activation in neuropathic pain states. *European journal of pain* 13:263-272.
- Thome M, Hofmann K, Burns K, Martinon F, Bodmer JL, Mattmann C, Tschopp J (1998) Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr Biol* 8:885-888.
- Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356:768-774.
- Todd AJ (2010) Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci* 11:823-836.

- Tofaris GK, Patterson PH, Jessen KR, Mirsky R (2002) Denervated Schwann cells attract macrophages by secretion of leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 in a process regulated by interleukin-6 and LIF. *J Neurosci* 22:6696-6703.
- Tong YG, Wang HF, Ju G, Grant G, Hokfelt T, Zhang X (1999) Increased uptake and transport of cholera toxin B-subunit in dorsal root ganglion neurons after peripheral axotomy: possible implications for sensory sprouting. *J Comp Neurol* 404:143-158.
- Torii H, Hosoi J, Beissert S, Xu S, Fox FE, Asahina A, Takashima A, Rook AH, Granstein RD (1997) Regulation of cytokine expression in macrophages and the Langerhans cell-like line XS52 by calcitonin gene-related peptide. *Journal of leukocyte biology* 61:216-223.
- Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K (2008) P2Y₁₂ receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *J Neurosci* 28:4949-4956.
- Tracey I, Mantyh PW (2007) The cerebral signature for pain perception and its modulation. *Neuron* 55:377-391.
- Trang T, Beggs S, Salter MW (2012) ATP receptors gate microglia signaling in neuropathic pain. *Experimental neurology* 234:354-361.
- Trang T, Beggs S, Wan X, Salter MW (2009) P2X₄-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. *J Neurosci* 29:3518-3528.
- Treede RD, Jensen TS, Campbell JN, Cruccu G, Dostrovsky JO, Griffin JW, Hansson P, Hughes R, Nurmikko T, Serra J (2008) Neuropathic pain: redefinition and a grading system for clinical and research purposes. *Neurology* 70:1630-1635.
- Trinchieri G, Sher A (2007) Cooperation of Toll-like receptor signals in innate immune defence. *Nature reviews Immunology* 7:179-190.
- Tsuda M, Inoue K, Salter MW (2005) Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends in neurosciences* 28:101-107.
- Tsuda M, Kuboyama K, Inoue T, Nagata K, Tozaki-Saitoh H, Inoue K (2009a) Behavioral phenotypes of mice lacking purinergic P2X₄ receptors in acute and chronic pain assays. *Mol Pain* 5:28.
- Tsuda M, Masuda T, Kitano J, Shimoyama H, Tozaki-Saitoh H, Inoue K (2009b) IFN-gamma receptor signaling mediates spinal microglia activation driving neuropathic pain. *Proc Natl Acad Sci U S A* 106:8032-8037.

- Tsuda M, Mizokoshi A, Shigemoto-Mogami Y, Koizumi S, Inoue K (2004) Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. *Glia* 45:89-95.
- Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K (2003) P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 424:778-783.
- Tsuda M, Ueno H, Kataoka A, Tozaki-Saitoh H, Inoue K (2008) Activation of dorsal horn microglia contributes to diabetes-induced tactile allodynia via extracellular signal-regulated protein kinase signaling. *Glia* 56:378-386.
- Uceyler N, Schafer M, Sommer C (2009) Mode of action of cytokines on nociceptive neurons. *Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale* 196:67-78.
- Uceyler N, Sommer C (2008) Cytokine regulation in animal models of neuropathic pain and in human diseases. *Neurosci Lett* 437:194-198.
- Ulmann L, Hatcher JP, Hughes JP, Chaumont S, Green PJ, Conquet F, Buell GN, Reeve AJ, Chessell IP, Rassendren F (2008) Up-regulation of P2X4 receptors in spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain. *J Neurosci* 28:11263-11268.
- Ulmann L, Hirbec H, Rassendren F (2010) P2X4 receptors mediate PGE2 release by tissue-resident macrophages and initiate inflammatory pain. *The EMBO journal* 29:2290-2300.
- Vallejo R, Tilley DM, Vogel L, Benyamin R (2010) The role of glia and the immune system in the development and maintenance of neuropathic pain. *Pain practice : the official journal of World Institute of Pain* 10:167-184.
- van de Loo FA, Bennink MB, Arntz OJ, Smeets RL, Lubberts E, Joosten LA, van Lent PL, Coenen-de Roo CJ, Cuzzocrea S, Segal BH, Holland SM, van den Berg WB (2003) Deficiency of NADPH oxidase components p47phox and gp91phox caused granulomatous synovitis and increased connective tissue destruction in experimental arthritis models. *The American journal of pathology* 163:1525-1537.
- Van Steenwinckel J, Reaux-Le Goazigo A, Pommier B, Mauborgne A, Dansereau MA, Kitabgi P, Sarret P, Pohl M, Melik Parsadaniantz S (2011) CCL2 released from neuronal synaptic vesicles in the spinal cord is a major mediator of local inflammation and pain after peripheral nerve injury. *J Neurosci* 31:5865-5875.
- Vanegas H, Schaible HG (2004) Descending control of persistent pain: inhibitory or facilitatory? *Brain Res Brain Res Rev* 46:295-309.

- Verge GM, Milligan ED, Maier SF, Watkins LR, Naeve GS, Foster AC (2004) Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. *Eur J Neurosci* 20:1150-1160.
- Wagner R, Myers RR (1996) Schwann cells produce tumor necrosis factor alpha: expression in injured and non-injured nerves. *Neuroscience* 73:625-629.
- Wallace VC, Blackbeard J, Pheby T, Segerdahl AR, Davies M, Hasnie F, Hall S, McMahon SB, Rice AS (2007) Pharmacological, behavioural and mechanistic analysis of HIV-1 gp120 induced painful neuropathy. *Pain* 133:47-63.
- Wang L, Manji GA, Grenier JM, Al-Garawi A, Merriam S, Lora JM, Geddes BJ, Briskin M, DiStefano PS, Bertin J (2002) PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing. *J Biol Chem* 277:29874-29880.
- Wang Y, Hasegawa M, Imamura R, Kinoshita T, Kondo C, Konaka K, Suda T (2004a) PYNOD, a novel Apaf-1/CED4-like protein is an inhibitor of ASC and caspase-1. *International immunology* 16:777-786.
- Wang ZQ, Porreca F, Cuzzocrea S, Galen K, Lightfoot R, Masini E, Muscoli C, Mollace V, Ndengele M, Ischiropoulos H, Salvemini D (2004b) A newly identified role for superoxide in inflammatory pain. *The Journal of pharmacology and experimental therapeutics* 309:869-878.
- Watanabe K, Konno S, Sekiguchi M, Sasaki N, Honda T, Kikuchi S (2007) Increase of 200-kDa neurofilament-immunoreactive afferents in the substantia gelatinosa in allodynic rats induced by compression of the dorsal root ganglion. *Spine* 32:1265-1271.
- Watkins LR, Maier SF (1999) Implications of immune-to-brain communication for sickness and pain. *Proc Natl Acad Sci U S A* 96:7710-7713.
- Watkins LR, Maier SF (2003) Glia: a novel drug discovery target for clinical pain. *Nat Rev Drug Discov* 2:973-985.
- Watkins LR, Martin D, Ulrich P, Tracey KJ, Maier SF (1997) Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. *Pain* 71:225-235.
- Watkins LR, Wiertelak EP, Goehler LE, Smith KP, Martin D, Maier SF (1994) Characterization of cytokine-induced hyperalgesia. *Brain Res* 654:15-26.
- Waxman SG, Cummins TR, Dib-Hajj SD, Black JA (2000) Voltage-gated sodium channels and the molecular pathogenesis of pain: a review. *Journal of rehabilitation research and development* 37:517-528.
- Weber A, Wasiliew P, Kracht M (2010) Interleukin-1 (IL-1) pathway. *Science signaling* 3:cm1.

- Wen YR, Suter MR, Kawasaki Y, Huang J, Pertin M, Kohno T, Berde CB, Decosterd I, Ji RR (2007) Nerve conduction blockade in the sciatic nerve prevents but does not reverse the activation of p38 mitogen-activated protein kinase in spinal microglia in the rat spared nerve injury model. *Anesthesiology* 107:312-321.
- Williams RO (2004) Collagen-induced arthritis as a model for rheumatoid arthritis. *Methods in molecular medicine* 98:207-216.
- Winkelstein BA, Rutkowski MD, Sweitzer SM, Pahl JL, DeLeo JA (2001) Nerve injury proximal or distal to the DRG induces similar spinal glial activation and selective cytokine expression but differential behavioral responses to pharmacologic treatment. *J Comp Neurol* 439:127-139.
- Wodarski R, Clark AK, Grist J, Marchand F, Malcangio M (2009) Gabapentin reverses microglial activation in the spinal cord of streptozotocin-induced diabetic rats. *European journal of pain* 13:807-811.
- Wolf G, Gabay E, Tal M, Yirmiya R, Shavit Y (2006) Genetic impairment of interleukin-1 signaling attenuates neuropathic pain, autotomy, and spontaneous ectopic neuronal activity, following nerve injury in mice. *Pain* 120:315-324.
- Wood JN, Boorman JP, Okuse K, Baker MD (2004) Voltage-gated sodium channels and pain pathways. *J Neurobiol* 61:55-71.
- Woodbury CJ, Kullmann FA, McIlwraith SL, Koerber HR (2008) Identity of myelinated cutaneous sensory neurons projecting to nociceptive laminae following nerve injury in adult mice. *J Comp Neurol* 508:500-509.
- Woolf CJ, Ma Q (2007) Nociceptors--noxious stimulus detectors. *Neuron* 55:353-364.
- Woolf CJ, Ma QP, Allchorne A, Poole S (1996) Peripheral cell types contributing to the hyperalgesic action of nerve growth factor in inflammation. *J Neurosci* 16:2716-2723.
- Woolf CJ, Shortland P, Coggeshall RE (1992) Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 355:75-78.
- Wu G, Ringkamp M, Murinson BB, Pogatzki EM, Hartke TV, Weerahandi HM, Campbell JN, Griffin JW, Meyer RA (2002) Degeneration of myelinated efferent fibers induces spontaneous activity in uninjured C-fiber afferents. *J Neurosci* 22:7746-7753.
- Wu J, Fernandes-Alnemri T, Alnemri ES (2010) Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by *Listeria monocytogenes*. *Journal of clinical immunology* 30:693-702.
- Xiao WH, Bennett GJ (2007) Persistent low-frequency spontaneous discharge in A-fiber and C-fiber primary afferent neurons during an inflammatory pain condition. *Anesthesiology* 107:813-821.

- Xie W, Strong JA, Zhang JM (2009) Early blockade of injured primary sensory afferents reduces glial cell activation in two rat neuropathic pain models. *Neuroscience* 160:847-857.
- Xu JJ, Walla BC, Diaz MF, Fuller GN, Gutstein HB (2006) Intermittent lumbar puncture in rats: a novel method for the experimental study of opioid tolerance. *Anesth Analg* 103:714-720.
- Xu ZZ, Berta T, Ji RR (2012) Resolvin E1 Inhibits Neuropathic Pain and Spinal Cord Microglial Activation Following Peripheral Nerve Injury. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*.
- Yajima Y, Narita M, Shimamura M, Narita M, Kubota C, Suzuki T (2003) Differential involvement of spinal protein kinase C and protein kinase A in neuropathic and inflammatory pain in mice. *Brain Res* 992:288-293.
- Yamamoto M, Yaginuma K, Tsutsui H, Sagara J, Guan X, Seki E, Yasuda K, Yamamoto M, Akira S, Nakanishi K, Noda T, Taniguchi S (2004) ASC is essential for LPS-induced activation of procaspase-1 independently of TLR-associated signal adaptor molecules. *Genes to cells : devoted to molecular & cellular mechanisms* 9:1055-1067.
- Yamamoto W, Sugiura A, Nakazato-Imasato E, Kita Y (2008) Characterization of primary sensory neurons mediating static and dynamic allodynia in rat chronic constriction injury model. *The Journal of pharmacy and pharmacology* 60:717-722.
- Yoo NJ, Park WS, Kim SY, Reed JC, Son SG, Lee JY, Lee SH (2002) Nod1, a CARD protein, enhances pro-interleukin-1 β processing through the interaction with pro-caspase-1. *Biochemical and biophysical research communications* 299:652-658.
- Yu JW, Wu J, Zhang Z, Datta P, Ibrahimi I, Taniguchi S, Sagara J, Fernandes-Alnemri T, Alnemri ES (2006) Cryopyrin and pyrin activate caspase-1, but not NF- κ B, via ASC oligomerization. *Cell Death Differ* 13:236-249.
- Zeitz KP, Guy N, Malmberg AB, Dirajlal S, Martin WJ, Sun L, Bonhaus DW, Stucky CL, Julius D, Basbaum AI (2002) The 5-HT₃ subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors. *J Neurosci* 22:1010-1019.
- Zhang J, De Koninck Y (2006) Spatial and temporal relationship between monocyte chemoattractant protein-1 expression and spinal glial activation following peripheral nerve injury. *J Neurochem* 97:772-783.
- Zhang J, Shi XQ, Echeverry S, Mogil JS, De Koninck Y, Rivest S (2007) Expression of CCR2 in both resident and bone marrow-derived microglia plays a critical role in neuropathic pain. *J Neurosci* 27:12396-12406.

- Zhang L, Berta T, Xu ZZ, Liu T, Park JY, Ji RR (2011) TNF- α contributes to spinal cord synaptic plasticity and inflammatory pain: distinct role of TNF receptor subtypes 1 and 2. *Pain* 152:419-427.
- Zhang RX, Li A, Liu B, Wang L, Ren K, Zhang H, Berman BM, Lao L (2008a) IL-1ra alleviates inflammatory hyperalgesia through preventing phosphorylation of NMDA receptor NR-1 subunit in rats. *Pain* 135:232-239.
- Zhang W, Liu LY, Xu TL (2008b) Reduced potassium-chloride co-transporter expression in spinal cord dorsal horn neurons contributes to inflammatory pain hypersensitivity in rats. *Neuroscience* 152:502-510.
- Zhang WH, Wang X, Narayanan M, Zhang Y, Huo C, Reed JC, Friedlander RM (2003) Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. *Proc Natl Acad Sci U S A* 100:16012-16017.
- Zhao J, Seereeram A, Nassar MA, Levato A, Pezet S, Hathaway G, Morenilla-Palao C, Stirling C, Fitzgerald M, McMahon SB, Rios M, Wood JN, London Pain C (2006) Nociceptor-derived brain-derived neurotrophic factor regulates acute and inflammatory but not neuropathic pain. *Mol Cell Neurosci* 31:539-548.
- Zheng FY, Xiao WH, Bennett GJ (2011) The response of spinal microglia to chemotherapy-evoked painful peripheral neuropathies is distinct from that evoked by traumatic nerve injuries. *Neuroscience* 176:447-454.
- Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 11:136-140.
- Zhou R, Yazdi AS, Menu P, Tschopp J (2011) A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469:221-225.
- Zhou XF, Chie ET, Deng YS, Zhong JH, Xue Q, Rush RA, Xian CJ (1999) Injured primary sensory neurons switch phenotype for brain-derived neurotrophic factor in the rat. *Neuroscience* 92:841-853.
- Zhuang ZY, Gerner P, Woolf CJ, Ji RR (2005) ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 114:149-159.
- Zhuang ZY, Kawasaki Y, Tan PH, Wen YR, Huang J, Ji RR (2007) Role of the CX3CR1/p38 MAPK pathway in spinal microglia for the development of neuropathic pain following nerve injury-induced cleavage of fractalkine. *Brain, behavior, and immunity* 21:642-651.
- Zhuang ZY, Wen YR, Zhang DR, Borsello T, Bonny C, Strichartz GR, Decosterd I, Ji RR (2006) A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons

and spinal astrocytes for neuropathic pain development and maintenance. *J Neurosci* 26:3551-3560.

Zuo Y, Perkins NM, Tracey DJ, Geczy CL (2003) Inflammation and hyperalgesia induced by nerve injury in the rat: a key role of mast cells. *Pain* 105:467-479.